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Molecular Genetics and Pathogenesis of *Clostridium perfringens*

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INTRODUCTION

The genus *Clostridium* consists of a diverse group of gram-positive bacteria which do not grow in the presence of oxygen and have the ability to form heat-resistant endospores. Many of these anaerobes are pathogenic for both humans and other animals, and most of the resultant diseases, such as tetanus and botulism, are mediated by the production of potent extracellular toxins. The ability to form spores is often an important factor in the epidemiology of these toxemias.

Clostridium perfringens is commonly found in the gastrointestinal tract of both humans and other animals, as well as in soil and sewage. *C. perfringens* has been shown to be a cause of human diseases such as gas gangrene (clostridial myonecrosis), food poisoning, necrotizing enterocolitis of infants, and enteritis necroticans (pigbel) (124, 194). It is also the causative agent of animal diseases such as lamb dysentery, ovine enterotoxemia (struck) and pulpy kidney disease of sheep, and other enterotoxemic diseases of lambs and calves (142, 197). Isolates of *C. perfringens* can be divided into five types (A to E) based on the particular extracellular toxins which they produce (Table 1). Each of these toxin types is responsible for specific disease syndromes (Table 2). Further descriptions of the many different extracellular enzymes and toxins made by different strains of *C. perfringens* can be found in the reviews by McDonel (124, 125). An excellent historical account of histotoxic clostridial infections is given in the review by MacLennan (104).

C. perfringens diseases are generally mediated via the production of extracellular enzymes or toxins, with the exception of human food poisoning, which involves a sporulation-specific enterotoxin. The toxins implicated in gas gangrene, the most serious of these diseases, are a phospholipase C (α-toxin) and a thiol-activated hemolysin (θ-toxin or perfringolysin O). The role in pathogenesis of a variety of other extracellular enzymes such as collagenase (κ-toxin), hyaluronidase (μ-toxin), DNase (ν-toxin), and neuraminidase (sialidase) remains to be precisely elucidated. The major toxin implicated in pulpy kidney disease is the potent ε-toxin, whereas lamb dysentery and human enteritis necroticans are primarily due to the effects of the β-toxin. The primary factor involved in *C. perfringens* food poisoning is an enterotoxin which traditionally has been thought of as a sporulation-related protein (121, 124, 194).

C. perfringens is different from many other clostridia in that it is nonmotile and, in vitro, forms spores only in specialized culture media. The organism is fermentative and grows rapidly in media containing carbohydrates. Under these conditions it produces copious amounts of H_2 and CO_2, which help to maintain an anaerobic environment. Owing to its rapid growth and relative aerotolerance, *C. perfringens* is easy to work with in the laboratory and has become a model organism for the development of clostridial genetics (231).

In recent years there have been rapid advances in the genetics of *C. perfringens*. A series of conjugative R plasmids have been mapped and characterized (2, 3, 6, 18, 106), several antibiotic resistance genes have been cloned and sequenced (2, 6, 9, 12, 166, 196), transposable genetic elements have been identified (4, 5), a bacteriocin-encoding plasmid has been analyzed in detail and completely sequenced (45–47, 49), and a physical and genetic map of the *C. perfringens* genome has been constructed (24). In addition, reproducible electroporation methods for the transformation of vegetative *C. perfringens* cells with plasmid DNA have been reported and are in routine use in several laboratories (7, 182). These developments, coupled with the construction of several *C. perfringens-Escherichia coli* shuttle plasmids (159, 191a, 195) and the cloning and sequencing of several *C. perfringens* toxin genes (69, 83, 161, 208, 214, 215, 218), have opened the way for detailed genetic studies on the pathogenesis of *C. perfringens* infections and have prompted the preparation of this review. Our objectives are to review the latest developments in this rapidly moving field and to convince the reader that molecular genetic approaches to the study of *C. perfringens* pathogenesis will yield exciting results in the not too distant future.

TABLE 1. Toxins produced by *C. perfringens*[a]

C. perfringens type	Toxins produced											
	α	β	ε	ι	δ	θ	κ	λ	μ	ν	Nm[b]	En[c]
A	+++	−	−	−	−	+	+	−	+	+	+	+
B	+	++	+	−	+	+	+	+	+	+	+	+
C	+	++	−	−	+	+	+	−	+	+	+	+
D	+	−	++	−	−	+	+	+	+	+	+	+
E	+	−	−	+	−	+	+	+	−	+	+	+

[a] Based on previous tables (104, 124).
[b] Nm, neuraminidase or sialidase.
[c] En, enterotoxin.

TABLE 2. Diseases caused by *C. perfringens*[a]

C. pergringens type	Disease produced
A	Gas gangrene (clostridial myonecrosis), food poisoning, necrotic enteritis of infants, necrotic enteritis of poultry
B	Lamb dysentery, enterotoxemia of sheep, foals, and goats
C	Enterotoxemia of sheep (struck), necrotic enteritis in animals, human enteritis necroticans (pigbel)
D	Enterotoxemia of sheep (pulpy kidney disease)
E	Enteritis of rabbits

[a] Based on several reviews (124, 142, 197).

BACTERIOPHAGES AND BACTERIOCINS

Bacteriophages of *C. perfringens*

Like most other species of bacteria, *C. perfringens* is susceptible to infection by bacteriophages. However, these phages have played very little role in the development of *C. perfringens* genetics. Early studies followed the same general approach as research on bacteriophages of other bacteria and were concerned mainly with the identification and classification of different morphological phage types. Phage-typing schemes of limited epidemiological value were developed, whereas attempts to correlate toxin production with the presence of particular phages were unsuccessful. Since the emphasis of this review is the genetics of *C. perfringens*, the reader is referred to other sources for details of the structure, general properties, and morphology of *C. perfringens* phages (109, 144).

Smith (193) examined 152 strains, of all toxin types, for the presence of lysogenic phages and for their susceptibility to phages isolated from sewage, feces, and other sources. Some 32 strains were lysogenic as tested by their ability to produce phage which could lyse and replicate in indicator

strains. In addition, a variety of phages were isolated from the environment. Several viruses appeared to be specific for indicator strains of particular toxin types. Since many of the *C. perfringens* strains were not susceptible to any of these phages, it is clear from this research and other studies, that *C. perfringens* bacteriophages have little epidemiological value (109). Four temperate phages, representing two distinct classes, were isolated from strains of *C. perfringens* type C, and unsuccessful attempts were made to obtain transduction of erythromycin resistance (54). Other workers analyzed a temperate *C. perfringens* phage, designated s9, and used the type A strain, Le chien, as an indicator (115). This phage recipient, more commonly known as strain 13, is now the most readily transformable *C. perfringens* strain, probably because it appears to lack a restriction system (182). Although this strain is capable of being lysogenized (115), virtually no attempts have been made to use transduction of strain 13 derivatives as a means of strain construction in *C. perfringens*.

Further experiments were carried out on the UV-inducible bacteriophage s9 (115) and on a second phage isolated in the same laboratory (114). A strain 13 derivative lysogenized with s9, as well as lysogenized and cured derivatives of the original lysogenic parent strain, was tested for the ability to sporulate (202). The results indicated that the lysogens produced heat-resistant spores more rapidly than did phage-free derivatives (3 to 4 h compared with 8 to 10 h). The percentage of refractile spores that were heat resistant appeared to increase significantly on lysogeny. It was suggested that this was an example of phage conversion, with the phage s9 presumably carrying a gene(s) involved in sporulation (202). Confirmation of this conclusion awaits modern molecular studies. Nonetheless, it appears to be the only report of phage-mediated phenotypic changes in *C. perfringens*.

The results of experiments involving the bacteriophage r infection of a series of strain NCTC 8798 sporulation mutants were also used to postulate the existence of a *C. perfringens* restriction and modification system (33). The results of this study imply that the wild-type strain carries a gene coding for a defective restriction endonuclease which can be mutated to an active form. It would be interesting to reexamine this system by using modern molecular methods of analysis. However, it is clear from both conjugation (163) and transformation (182) studies, and the susceptibility of DNA to cleavage by certain restriction endonucleases (44), that restriction and modification systems do exist in *C. perfringens*. Preliminary studies have led to the identification of a restriction endonuclease which is an isoschizomer of *Mbo*I (140a).

Early studies showed that the bacteriocinogenic *C. perfringens* BP6K-N5 (now known as CPN50) carried a lysogenic phage, φ29 (82). The most recent work on *C. perfringens* phages involved the mapping of the chromosomal attachment sites of φ29 and another lysogenic phage, φ59. Pulsed-field gel electrophoresis was used to compare wild-type and lysogenic derivatives of strain CPN50. The results showed that the φ59 attachment site was located near the *Mlu*I site at 2.9 Mb on the CPN50 map and that the φ29 site was near the *Mlu*I site at 1.0 Mb (25). The φ29 site was located within a 180-kb *Fsp*I fragment, which also contains the *nanH* gene, which encodes neuraminidase (sialidase) production (161). The φ59 site was located near the *atpD* gene, which codes for ATP synthase (24).

Although phages of *C. perfringens* have been known for many years, there have been no reports of the transduction of either chromosomal or plasmid-determined genes. Similarly, unlike the situation for many other clostridial species, there is no solid evidence that any *C. perfringens* toxin genes, or other virulence factors, are phage determined. Although phage resistance has proven to be a useful marker for the genetic analysis of transconjugants (18), studies of bacteriophage genetics in *C. perfringens* have been unrewarding.

Bacteriocinogenic Strains of *C. perfringens*

Many *C. perfringens* isolates have been shown to produce bacteriocins capable of lysing indicator strains of *C. perfringens* (see the review by Tagg et al. [207] for a list of early papers). *C. perfringens* bacteriocins have proven to be more useful than bacteriophages for the development of typing schemes, and the analysis of bacteriocin-encoding plasmids has played a very important role in the development of *C. perfringens* genetics.

Several studies which have examined the ability of *C. perfringens* strains to produce bacteriocins have been carried out (107, 111). On the basis of the results, a *C. perfringens* typing scheme which relied on testing the susceptibility of strains to 10 distinct bacteriocins was developed (107). Analysis of the susceptibility of 274 strains of *C. perfringens* to these bacteriocins enabled them to be placed into seven bacteriocin types and 50 subtypes. Only three strains were untypable (107). Although subsequent studies have used this scheme to type clinical isolates of *C. perfringens* (119), it is not generally in widespread or routine use in clinical laboratories. An alternative bacteriocin-typing scheme has also been reported (177), and further developments of the original scheme have also been made (179). Other studies have focused on the mechanism of action, the production, or the characterization of *C. perfringens* bacteriocins (28, 81, 100, 108, 110, 112, 116).

PLASMIDS

Bacteriocin Plasmids: an Overview

C. perfringens CPN50 not only carries the bacteriophage φ29 but also produces a bacteriocin, known as N5 or BCN5, upon UV induction. Cured derivatives of CPN50 which no longer produce bacteriocin were isolated after acriflavine treatment and shown to be lacking plasmid DNA (80). Electron-microscopic analysis showed that the loss of both bacteriocin production and immunity was associated with the loss of a small (5.7-MDa) plasmid (79). Studies of strains 28 (99) and 55 (129) also showed a relationship between these phenotypic properties and small (5.6-MDa) plasmids. Since bacteriocin production in all three strains was associated with plasmids of the same size, it is tempting to assume that these plasmids are very similar (129). However, no comparative restriction studies have been done. The plasmid carried by strain 55, pCW4, was nonconjugative, whereas transfer of pIP404, the bacteriocin plasmid from CPN50, has been reported (18). Mobilization by a large conjugative plasmid present in strain CPN50 (79) is the most likely explanation for these results.

Bacteriocin Plasmid pIP404

Plasmid pIP404 can be considered a paradigm plasmid analogous to ColE1 in *E. coli*. It has been completely mapped and sequenced and is the best-studied *C. perfrin-*

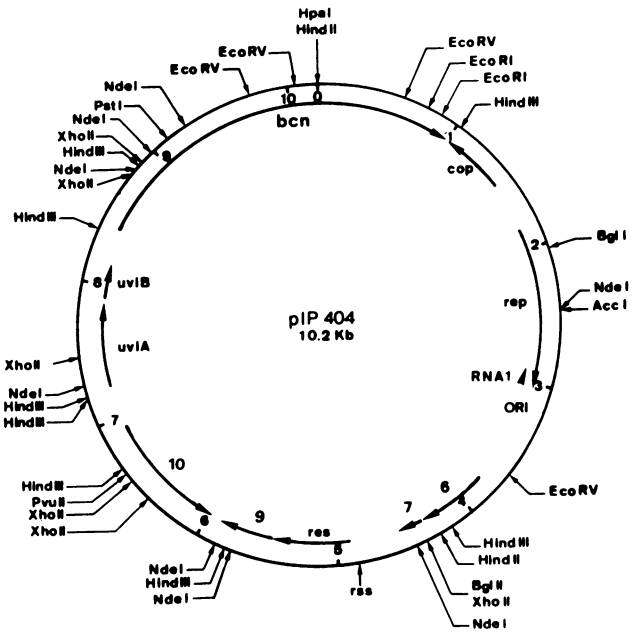

FIG. 1. Organisation and restriction map of the bacteriocinogenic plasmid pIP404. The positions and sizes of the genes are indicated by arrows. Known genes and their products are as follows; *uviA*, possible bacteriocin immunity protein; *uviB*, possible bacteriocin secretion protein; *bcn*, bacteriocin BCN5; *cop*, copy number control; *rep*, DNA replicase; *RNA1*, antisense RNA to *rep*, involved in copy number control; *res*, resolvase involved in site-specific recombination; *rss*, possible resolution site for resolvase. No functions have been attributed yet to open reading frames 6, 7, 9, and 10. ORI denotes the putative origin of replication. Based on previous data (45).

gens plasmid (44–47, 49). The 10,207-bp genome of pIP404 has a typical clostridial dA+dT content (75%) and consists of 10 open reading frames and a complex locus corresponding to the putative origin of replication, *ori* (45, 46). Figure 1 summarizes our current knowledge of the genetic organization of pIP404, and Table 3 presents the salient properties of the gene products. Basically, the genes can be classified into two groups, one involved in plasmid replication and maintenance and the other associated with bacteriocin production.

Replication of pIP404. To identify genetic loci required for replication of pIP404, overlapping restriction fragments were cloned into a vector which carried a selectable marker, but no origin of replication, and were used to transform *Bacillus subtilis*. Subsequently, when transformation of *C. perfringens* became possible, the properties of the resultant hybrid plasmids were confirmed by using the homologous host. In this way the minimal origin of replication was defined and shown to consist of the *rep* gene, which encodes a 48.7-kDa basic DNA-binding protein (Table 3), and *ori*. The latter locus is composed of two related families of direct repeats, X and Y, where X is TAAAAAAATATAAAAA and Y is TAAAAAAA, arranged as a dispersed tandem array. In this region there is a 222-bp segment consisting entirely of dA+dT, which can be summarized as $Y_{12}X_4YXYX$. This arrangement of repeated sequences is highly reminiscent of the origins of replication of numerous plasmids and phages (180). Detailed deletion analysis showed that essentially all of this locus was required for replication of pIP404 (46).

The majority of plasmids from gram-positive bacteria replicate via the rolling-circle method and use a single-stranded intermediate (65). When used as cloning vectors, such plasmids display pronounced physical instability, as the inserted fragments often carry fortuitous minus origins of replication and are prone to deletions and recombinational rearrangements. Fortunately, the pIP404 replicon does not employ a single-stranded replicative intermediate, either in *C. perfringens* or in *B. subtilis*, and most probably uses the theta method of replication (84). As a result, vectors based on pIP404 should exhibit greater stability, and, to date, no deleterious genetic events have been observed with derivatives carrying foreign DNA (29a, 164a).

Control of replication and plasmid maintenance. At least three different means of copy number control are apparently used by pIP404 (46, 49). The first of these is effected by an antisense RNA molecule, RNA1, that consists of 150 nucleotides and is produced in large amounts by a powerful promoter, PA1 (Fig. 2b) situated within *ori* (46), (Fig. 1). The RNA1 transcriptional unit overlaps the 3′ end of the *rep* gene by ca. 124 bp and is complementary to the part of the *rep* mRNA which encodes the putative DNA-binding domain. Consequently, rounds of replication initiation could be limited by the availability of the Rep protein, stemming from antisense sequestering of its mRNA.

The second means of copy number control results from the action of the *cop* gene, which is situated adjacent to *rep* (Fig. 1). This gene encodes a 23,361-Da, tyrosine-rich, hydrophobic protein which is predicted to be localized to the cell membrane (Table 3). The absence of the Cop protein

TABLE 3. Gene-product relationships for pIP404

Gene	Probable function of product	Location of product	Mol wt of product	
			Predicted	Observed[a]
bcn	BCN5 bacteriocin, probably ionophoric	Extracellular	96,591	96,000, 80,000
uviA	Probable immunity protein	Cytoplasmic	22,013	22,000
uviB	Bacteriocin secretion	Membrane bound	7,607	NF
rep	DNA replication, DNA binding	Cytoplasmic	48,712	NF
cop	Copy number control	Membrane bound	23,262	NF
orf6	Unknown	Cytoplasmic	19,703	20,500
res	Multimer resolution, DNA binding, copy number control	Cytoplasmic	6,074	NF
orf7	Unknown	Cytoplasmic	21,403	NF
orf9	Unknown	Cytoplasmic	14,498	NF
orf10	Unknown	Cytoplasmic	38,321	NF
RNA1	Antisense RNA, 150 bases, replication control	Cytoplasmic		

[a] NF, gene product not found.

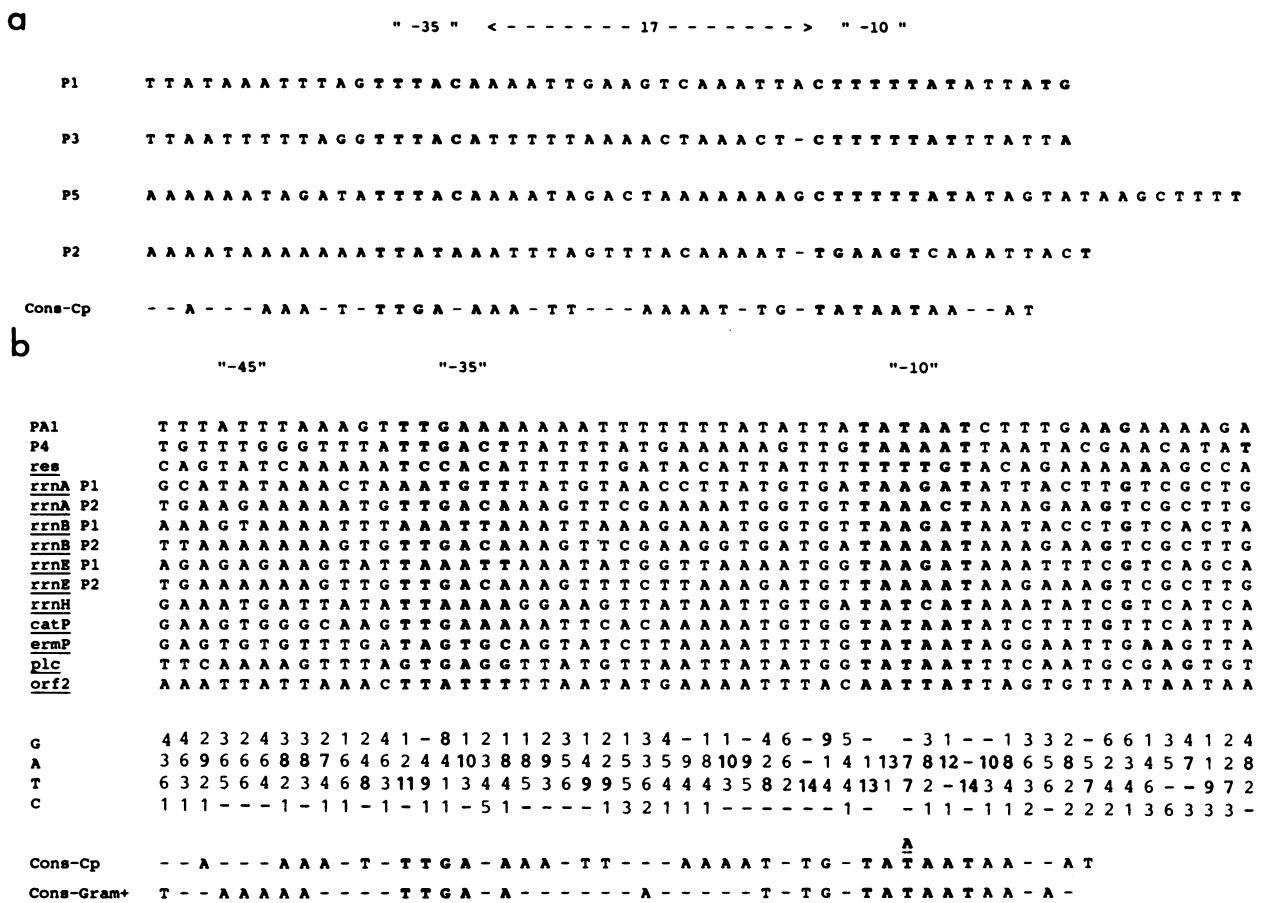

FIG. 2. Nucleotide sequence of *C. perfringens* promoters. (a) Structure of four UV-inducible promoters (P1, P2, P3, and P5) from pIP404 and comparison with the consensus sequence for a *C. perfringens* promoter. The −35 and −10 regions, as well as the transcriptional start points, are shown in boldface type (47). (b) Structure of 14 well-characterized promoters recognized by the major form of RNA polymerase and the deduction of a consensus sequence. The sources of the promoters were PA1 and P4 (47); *res* (49); *rrnA*, *rrnB*, *rrnE*, and *rrnH* (43); *catP* (196); *ermP* (120); and *plc* and *orf2* (176). The frequency of occurrence of individual nucleotides was tabulated, and when a given nucleotide appeared with a frequency of at least 50%, this was used to deduce the consensus *C. perfringens* (Cons-Cp) promoter sequence, which is compared with the extended promoter sequence for a gram-positive bacterium (Cons-Gram+ [63]).

leads to a fivefold increase in the copy number of pIP404-based vectors in *B. subtilis* (46). The effect of *cop* in *C. perfringens* is less pronounced, and *cop*$^+$ derivatives appear to be present in 10 to 15 copies per cell (i.e., like pIP404), whereas *cop* clones have an apparent copy number of 20 to 25 (47a).

An additional means of maintaining copy number control and ensuring efficient distribution of plasmid molecules to daughter cells during cell division is provided by the *res* gene of pIP404, which encodes a resolvase highly homologous to the site-specific recombinases of various transposons and phages (49). As for the gamma delta resolvase (64), the promoter of the *res* gene is overlapped by three potential resolution sites which could be used as resolvase substrates for the resolution of multimers of pIP404, which result from homologous recombination, into the monomeric form. Although the effect of *res* on the stability of pIP404-based vectors has not yet been evaluated, it is quite likely to be beneficial. Additional support for this hypothesis is provided by the recent discovery of a gene analogous to *res* on the broad-host-range plasmid pAMβ1 from *Enterococcus faecalis* (130a).

Bacteriocin production and immunity. Ionesco et al. (82) showed that *C. perfringens* CPN50 secreted the bacteriocin BCN5 into the medium following UV irradiation, which suggests control of DNA damage by an SOS-like system (221). To localize the *bcn* gene on pIP404, differential dot blot hybridization was performed with RNA prepared from cultures before and after UV irradiation (44, 47). This approach revealed that a 4-kb segment consisting of the *bcn* gene and the *uviAB* operon (Fig. 1) was heavily transcribed in response to DNA damage. Following activation of the *bcn* gene, copious amounts of a 96-kDa protein accumulate in the cytoplasm, to be released into the medium some 2 h after induction (31). The primary structure of BCN5 deduced from the nucleotide sequence reveals a protein of 890 amino acid residues (molecular mass, 96 kDa), with a high glycine content (11.5%) which is characteristic of bacteriocins and is believed to facilitate their transfer across cell membranes (153, 154). Although the exact biological activity of BCN5 is unknown, the presence of an extended lipophilic region near the COOH terminus suggests that it might function as an ionophore (44). At least part of the 96-kDa polypeptide appears to be converted to an 80-kDa form by proteolysis (44). At present we do not know whether both species have bacteriocin activity.

TABLE 4. Antibiotic resistance determinants from *C. perfringens*

Resistance determinant	Origin	Location	Resistance mechanism
Tet P	pCW3	Plasmid/chromosomal	Efflux of tetracycline
CAT P	pIP401	Plasmid	CAT
CAT Q	CW92	Chromosomal	CAT
Erm BP	CP592	Plasmid/chromosomal	rRNA methylase
Erm Q	CW459	Chromosomal	rRNA methylase

In addition to encoding BCN5, pIP404 codes for bacteriocin immunity (18), and a series of experiments suggests that the *uviA* gene is responsible for this activity. This gene is the promoter-proximal cistron of the UV-inducible *uviAB* operon, situated downstream of *bcn* (Fig. 1), and its product is a soluble protein of 22 kDa. In contrast, the *uviB* gene encodes a small protein of 64 residues, with a hydrophobic NH_2-terminal domain and a highly polar COOH terminus, that might play a role in BCN5 secretion by perturbing the cell membrane.

Transcription signals required for UV-inducible expression. To locate the promoters of the *bcn* and *uviAB* genes, a detailed transcriptional analysis was performed, as these genes could represent the prototypes of a putative clostridial SOS response and thus provide valuable regulatory insight. The mRNAs for both the *bcn* and *uviAB* transcriptional units were found to initiate at multiple, inducible promoters and to terminate after large stem-loop structures resembling factor-independent terminators (31). The *bcn* gene is transcribed from three promoters, P1, P2, and P3, whereas the *uviAB* operon is controlled by two promoters, P4 and P5. Of these five promoters only one, P4, bears any resemblance to the consensus clostridial promoter sequence (Fig. 2b) and can productively interact with purified vegetative RNA polymerase from *C. perfringens* (47, 48). We suspect that the role of P4 is to provide immunity functions through low-level transcription of *uviAB* during normal growth conditions. In contrast to promoter P2, which has a very unusual organization, promoters P1, P3, and P5 are highly similar and have identical −35 and −10 regions, where the sequences TTTA CA and CTTTTTAT occur, respectively (47) (Fig. 2a).

Conjugative Plasmids

C. perfringens is the only member of the genus *Clostridium* in which conjugative antibiotic resistance plasmids (R plasmids) have been found. All of these plasmids are closely related and encode the same tetracycline resistance determinant (3, 6), designated Tet P in accordance with accepted terminology (97). Some of these plasmids also carry a chloramphenicol resistance determinant, designated CAT P (Table 4) (6, 18).

A large number of conjugative R plasmids have been found in various strains of *C. perfringens* (3, 6, 18, 70, 168). Two of these plasmids, pIP401 and pCW3, have been extensively studied (2, 4, 6, 106) and shown to be closely related (6). Genetic analysis has shown that both pIP401 and pCW3 carry genes encoding their own transfer, which occurs by a conjugationlike process (18, 168, 183, 184). The 54-kb plasmid, pIP401, carries both the Tet P and CAT P determinants, whereas pCW3 (47 kb) confers only tetracycline resistance. Loss of chloramphenicol resistance from pIP401 is often observed after conjugative transfer and is associated with the loss of a 6.2-kb segment of DNA (6, 18). Subsequent studies showed that this segment comprises the

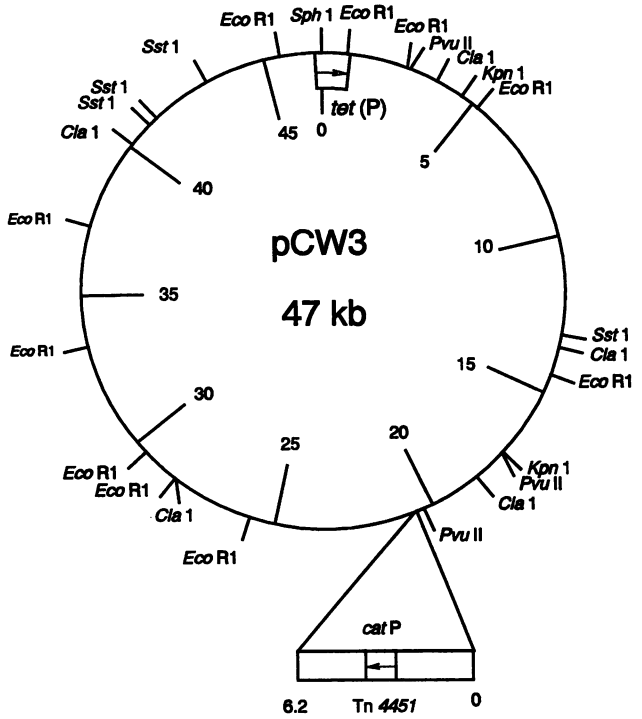

FIG. 3. Relationship between the conjugative R plasmids pCW3 and pIP401. The restriction map of pCW3 is drawn with the arbitrary zero point at the single *Sph*I site as before (2). The plasmid pIP401 consists of a pCW3 replicon that contains the 6.2-kb transposon Tn*4451* at the site indicated (4, 6). The location and direction of transcriptional of the *tet*(P) (1, 3) and *catP* (4, 6) genes are also indicated.

transposable element, Tn*4451* (4). A closely related plasmid, pJIR27, carries a very similar chloramphenicol resistance transposon, Tn*4452* (4, 6).

The entire pCW3 replicon has been cloned in *E. coli* by separately cloning each of the five *Cla*I fragments that are present in pCW3. Restriction maps were elucidated for each of these plasmids and, together with restriction data obtained from digests of pCW3, used to construct a detailed physical map of the entire plasmid (2). A restriction map of pIP401 has also been elucidated (106). Comparison of these maps reveals that these plasmids are very closely related (Fig. 3) and that two pIP401-derived, chloramphenicol-sensitive deletion plasmids (6, 106) are indistinguishable from pCW3 (6). The pIP401 replicon can therefore be considered to be a pCW3 plasmid which contains the chloramphenicol resistance transposon Tn*4451* (4, 6). However, it is not known whether pCW3 is a transposon deletion derivative of pIP401 or whether pIP401 is a transposon insertion derivative of pCW3.

In other studies, conjugative tetracycline-resistant strains of *C. perfringens* have been isolated from diverse sources, including human and animal feces and the environment (18, 70, 131, 132, 163, 167). Molecular analysis has shown that the conjugative plasmids harbored by all of these strains carry the Tet P resistance determinant. In addition, three of these plasmids carry the CAT P determinant (3, 6). No other phenotypic markers have been identified on any conjugative *C. perfringens* plasmids (163).

Molecular analysis of these conjugative plasmids and, in particular, comparison of their restriction profiles revealed

that they are all either identical to, or closely related to, the prototype plasmid, pCW3. Plasmids which could not be distinguished from pCW3 by restriction analysis were detected in transconjugants derived from porcine, bovine, human, and environmental strains from the United States, France, Belgium, Japan, and Australia (3, 6). All of the remaining conjugative plasmids that have been reported in the literature have at least 17 kb of restriction identity with pCW3 (3, 6, 70). The Tet P determinant is located within this region (2, 6).

Unlike the situation in most bacterial species, it is clear that in *C. perfringens* there is only one major type of conjugative R plasmid. All of the plasmids so far identified and analyzed carry the same tetracycline resistance determinant and were presumably derived either from pCW3 or from a common progenitor plasmid. Note that the sources of the strains from which these plasmids were isolated were extremely diverse, from both a geographical and an environmental perspective. It is concluded that there must be a worldwide gene pool of antibiotic-resistant *C. perfringens* strains, with ready distribution of plasmids within this gene pool. The migration patterns of both humans and other animals would be key factors contributing to the dispersal of R plasmids within and between these different environmental niches (6).

Other Plasmids

There are many reports in the literature of cryptic *C. perfringens* plasmids (3, 13, 14, 18, 70, 92, 113, 118, 149, 158, 162, 167, 168, 192). Plasmid profiling also has been used for strain differentiation in *C. perfringens* (118, 149).

Apart from the plasmids already mentioned, there are very few *C. perfringens* plasmids which confer defined phenotypes. Only one nonconjugative R plasmid has been identified, a large plasmid, pIP402, which confers resistance to the macrolide-lincosamide-streptogramin B (MLS) antibiotics (18). A small (3.1-kb) plasmid, pHB101, which confers caseinase, or λ-toxin, activity was identified several years ago in a type B strain of *C. perfringens* (14). Despite its small size, this plasmid has not been analyzed in detail and the toxin gene has not been cloned or sequenced. It is interesting that again, unlike the situation for other bacteria, no virulence plasmids have been clearly identified in *C. perfringens*, with the possible exception of pHB101. It has been suggested that the *C. perfringens* β-toxin gene may be located on a large plasmid, but this report remains unconfirmed (35).

ANTIBIOTIC RESISTANCE DETERMINANTS

Penicillin has traditionally been regarded as the drug of choice for the treatment of *C. perfringens* gas gangrene, and no β-lactamase-mediated penicillin-resistant strains of *C. perfringens* have been reported (224). However, doubts have been cast upon the efficacy of penicillins and cephalosporins in the clinical situation. Comparison of in vivo and in vitro effectiveness of several antimicrobial agents, including the penicillins, indicate some discrepancies between these tests. The treatment of *C. perfringens* infections therefore may not be as straightforward as is commonly believed, and other antibiotics, or combinations thereof, may be more suitable than the penicillins (198, 199, 211, 212). It follows that it is important that resistance levels to various antimicrobial agents be monitored routinely and that the mechanisms by which *C. perfringens* isolates mediate and disseminate their antimicrobial resistance be well understood.

The Tetracycline Resistance Determinant, Tet P

Tetracycline resistance is probably the most common antibiotic resistance phenotype found in *C. perfringens*. The majority of tetracycline-resistant strains do not have the ability to transfer their resistance. However, conjugative tetracycline resistance plasmids can be readily detected (18, 131, 132, 163, 165, 168). Tetracycline resistance in nonconjugative isolates is generally constitutively expressed, whereas it is inducible in conjugative strains (163). The molecular basis for this difference is not known, although it has been postulated that plasmids like pCW3 carry a gene which encodes a repressor of tetracycline resistance (2).

The tetracycline resistance determinant from pCW3, Tet P, has been cloned and shown to be located on two juxtaposed 1.9- and 2.1-kb *Eco*RI fragments on the pCW3 map (Fig. 3) (2, 195). Subcloning and transposon mutagenesis experiments have shown that the *Sph*I site at 0 kb and the *Eco*RI site at 0.8 kb (pCW3 coordinates) are both located within the Tet P determinant (1, 2). Preliminary DNA sequence data indicate that both sites are located within gene regions encoding hydrophobic domains typical of membrane proteins (191a). These results are in agreement with observations which indicate that the Tet P determinant mediates an active efflux of tetracycline (126a).

To determine the distribution of the Tet P determinant in various bacteria, the internal 0.8-kb *Sph*I-*Eco*RI fragment was purified and used as a gene-specific *tet*(P) hybridization probe in a series of Southern blot experiments. The results showed that the *tet*(P) gene, or a gene very closely related to it, was present in eight nonconjugative isolates of *C. perfringens*. It is clear that the same tetracycline resistance determinant is present in the constitutive nonconjugative isolates, where the resistance gene appears to be chromosomally determined, and in the inducible isolates which carry the *tet*(P) gene on conjugative pCW3-like plasmids (1). Additional studies showed that the *tet*(P) probe hybridized with a tetracycline-resistant strain of *Clostridium paraputrificum* but did not hybridize with a resistant *Clostridium sporogenes* isolate or six tetracycline-resistant *Clostridium difficile* strains. Thus, although the Tet P determinant is not restricted to *C. perfringens*, it is not widespread throughout the clostridia. Transfer of pIP401 from *C. perfringens* to *C. difficile* has been demonstrated, but the resultant tetracycline-resistant transconjugants were unstable (78). The fairly limited dissemination of the Tet P determinant may therefore be due to the narrow host range of pCW3-like plasmids.

In *C. difficile*, tetracycline resistance is mediated via a Tet M-like determinant, which is located on conjugative, chromosomally determined transposons similar to those found in the enterococci and streptococci (67, 137, 228). The enterococcal *tet*(M)-encoding transposon Tn*916* does transfer to *C. perfringens*, and the *tet*(M) gene is expressed (6a, 181a, 196a). However, no naturally occurring *C. perfringens* isolates which carry a Tet M-like determinant have been reported. Further screening of clinical isolates is required to see whether Tet P is the only naturally occurring class of tetracycline resistance determinant found in *C. perfringens*.

Several different classes of tetracycline resistance determinants have been identified in diverse bacterial species (97). Although many of these determinants mediate their resistance by an active efflux mechanism (96, 127), hybridization experiments failed to reveal any homology between these genes and *tet*(P) (1). The determination of the precise relationship between these genes awaits the elucidation of the complete nucleotide sequence of *tet*(P).

The Chloramphenicol Resistance Determinants, CAT P and CAT Q

Identification of the CAT P and CAT Q determinants. Chloramphenicol resistance is less common than either tetracycline or erythromycin resistance in *C. perfringens* (165, 167). Resistance is mediated by the production of chloramphenicol acetyltransferases (CAT) (Table 4) (9, 168, 234). Three conjugative tetracycline resistance plasmids (pIP401, pJIR25, and pJIR27) have been shown to carry chloramphenicol resistance determinants (6, 18), and two other strains have also been shown to harbor chloramphenicol resistance plasmids (166). Only one other chloramphenicol-resistant strain, CW92 or VPI11268, has been analyzed in detail. This isolate, which is the original strain from which pCW3 was obtained, carries the chromosomally determined *catQ* gene (166, 168).

The chloramphenicol resistance gene from pIP401, *catP*, has been cloned, localized, and shown to be expressed in *E. coli* (6). Hybridization analysis has shown that all five of the plasmid-determined *C. perfringens cat* genes share sequence similarity with *catP* (6, 166). However, the remaining chloramphenicol-resistant *C. perfringens* isolate that is available for analysis, CW92, did not hybridize with a *catP*-specific probe (166). The chloramphenicol resistance gene, *catQ*, from a derivative of this strain (168), has also been cloned and shown to be expressed in *E. coli* (166). Comparative restriction analysis and Southern blots confirmed that *catP* and *catQ* were different genes, even though both were of *C. perfringens* origin. Further hybridization studies showed that neither of these genes hybridized with *cat* genes from other genera (166). However, hybridization and sequence analysis has revealed that the *catD* gene from *C. difficile* is very closely related to the *catP* gene (196, 226, 227). The finding that essentially identical chloramphenicol resistance determinants are present in *C. perfringens* and *C. difficile* is in agreement with the results obtained for the respective erythromycin resistance determinants (11). In contrast, quite different tetracycline resistance genes are found in these species (1).

Conjugative transfer of pIP401 from *C. perfringens* to *C. difficile* has been reported, but the resultant unstable tetracycline-resistant transconjugants were not screened for chloramphenicol resistance (78). In addition, although the *catP* gene is carried within the pIP401-derived transposon Tn*4451* (4), the *catD* gene does not appear to be located on either a plasmid or a transposon (166, 226). The difference between the species distribution of the *catP* and *tet*(P) genes can be explained by postulating that conjugative transfer of pIP401-like plasmids from *C. perfringens* may be responsible for the introduction of both genes to *C. difficile*. However, the tetracycline resistance determinant may have been lost because the plasmid was unstable in *C. difficile* whereas the chloramphenicol resistance determinant was capable of transposing to the *C. difficile* chromosome, although the complete transposon presumably was lost at some later stage. If this hypothesis is correct, it may be possible to find either complete or partial copies of Tn*4451* in some chloramphenicol-resistant *C. difficile* strains.

Relationship between clostridial CAT enzymes. The nucleotide sequences of the *catP* (196), *catD* (227), and *catQ* (9) genes have been determined, and the amino acid sequences of the encoded CAT enzymes have been elucidated. These sequences have been compared with the amino acid sequences of 10 other CAT enzymes (9). All three clostridial CAT monomers shared considerable amino acid sequence identity with the other enzymes, including the regions known to be involved in enzyme activity (98, 186, 187). The *catP*- and *catD*-encoded enzymes have 98% amino acid sequence identity and are also the only CAT monomers which contain a 4-amino-acid deletion between residues 38 and 41 (196, 227). This deletion is not present in the CAT Q enzyme (9).

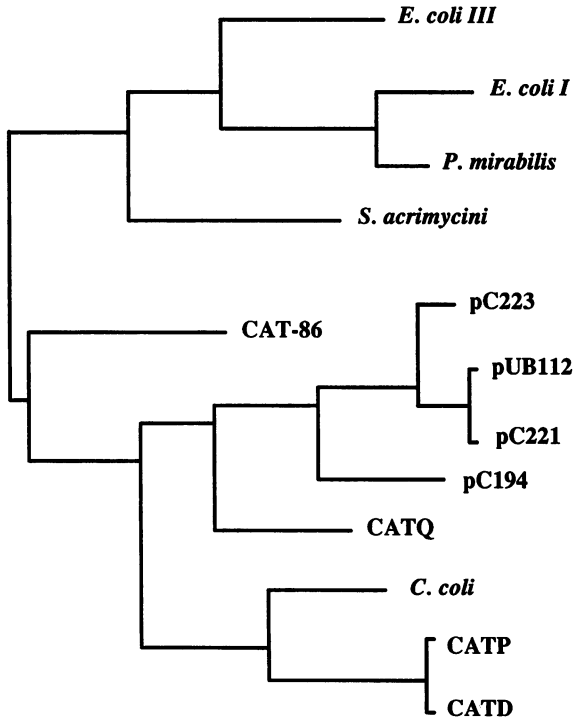

FIG. 4. Phylogenetic relationships between CAT monomers. A phylogenetic tree of CAT monomer amino acid sequences was prepared by using the parsimony method in conjunction with pairwise alignment. Designations refer to either the genetic locus, plasmid, or bacterial species of origin. Reproduced with permission from reference 9.

To obtain an insight into the evolutionary relationships between the CAT enzymes, their amino acid sequences were used to construct a phylogenetic tree (9). The resultant dendrogram (Fig. 4) revealed that the CAT Q monomer was as closely related to CAT enzymes from *Staphylococcus aureus* as it was to CAT P or CAT Q. Surprisingly, the *Campylobacter coli* CAT monomer (223) is more similar to CAT P and CAT D than the CAT Q enzyme is (9). It is clear from these results that the *catP* and *catD* genes are derived from a fairly recent, in evolutionary terms, common ancestor, whereas the *catQ* gene has evolved independently, albeit from a common primordial gene. The relationship of these genes to the *catA* and *catB* genes from *Clostridium butyricum* (34) remains to be determined, although hybridization analysis has shown that they are not closely related (8a).

Characterization of the transposons Tn*4451* and Tn*4452*. Analysis of the conjugative chloramphenicol resistance plasmids indicated that the chloramphenicol resistance determinants were often lost on conjugation and that an identical ca. 6-kb fragment was always deleted (6, 18, 184). In addition, the *catP* recombinant plasmids derived from pIP401 and pJIR27 were unstable in *recA* strains of *E. coli*, with identical

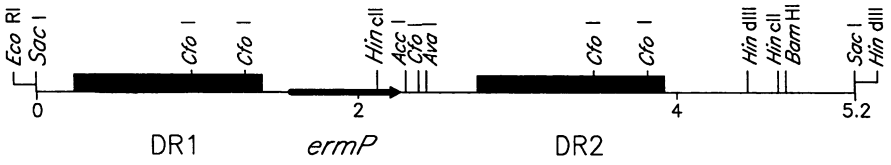

FIG. 5. Genetic organization of the *C. perfringens* Erm BP determinant. The restriction map of the *ermBP* gene region of the recombinant plasmid pJIR122 is shown. The *ermBP* gene is indicated by the arrow, and the direct repeats (DR1 and DR2) are shown by the dark boxes. Data were derived by D. Berryman and J. Rood and reproduced with permission from reference 164.

6.2-kb fragments being lost spontaneously (4). Detailed restriction and sequence analysis showed that the end products arising from events occurring in *C. perfringens* and *E. coli* were identical and that in each organism the deletion event was precise (4, 5). Confirmation that the 6.2-kb regions represented chloramphenicol resistance transposons was obtained for *E. coli* when *recA*-independent transposition of chloramphenicol resistance from temperature-sensitive plasmids to the chromosome was demonstrated (4). Heteroduplex analysis was done to compare the two transposons Tn*4451* and Tn*4452*, from pIP401 and pJIR27, respectively. The results revealed that they were very closely related, differing only in a 0.4-kb region at the right end of each transposon (4).

The mechanism(s) by which conjugative excision of Tn*4451* (and Tn*4452*) occurs in *C. perfringens* and spontaneous excision occurs in *E. coli* is not known. However, it is tempting to speculate that these events arise from part of the normal transposition process, perhaps involving a circular intermediate as in the enterococcal transposon Tn*916* (181).

Sequence analysis of the ends of Tn*4451* showed that the termini of the transposon contain small imperfect inverted repeats that have some similarity with the ends of the Tn*3* family. A CTAA sequence, which is often observed at the internal terminus of the 38-bp Tn*3* inverted repeat (188), and the terminal GGGGTC sequence are both present in Tn*4451* (5). However, Tn*4451* is not really a member of the Tn*3* family since preliminary evidence suggests that it may duplicate a 2-bp sequence upon insertion (5), whereas Tn*3* transposons have been shown to duplicate a 5-bp target sequence (188). Tn*4451*-R also contains an outward-firing −35 promoter consensus sequence close to the end of the transposon (5), which is typical of many transposable elements (42).

The Erythromycin Resistance Determinants, Erm BP and Erm Q

Erythromycin-resistant strains of *C. perfringens* are isolated more frequently than chloramphenicol-resistant derivatives. Resistance to erythromycin is always associated with resistance to clindamycin and lincomycin and is therefore referred to as MLS resistance (18, 32, 37, 38, 165, 167). The erythromycin resistance determinants are not located on conjugative R plasmids or on conjugative transposons, since in mixed-plate mating experiments resistance is not transferable (18, 63, 165). However, one nonconjugative erythromycin resistance plasmid, pIP402, has been identified (18).

The erythromycin resistance gene, designated *ermP*, from a strain carrying pIP402 has been cloned, localized, and shown to be expressed in *E. coli* (11). An intragenic probe was then used in a series of hybridization experiments to examine the distribution of the Erm P determinant. The results were very different from those obtained with the *tet* and *cat* probes. Only 5 of the 40 erythromycin-resistant *C. perfringens* strains tested hybridized with the *ermP* probe (11). The Erm P determinant is therefore not widely distributed in *C. perfringens*. We have recently cloned a second erythromycin resistance gene, *ermQ*, from a different strain of *C. perfringens* (Table 4) and have shown that it is distinct from *ermP* (102a).

Comparative hybridization analysis has shown that the *ermP* gene is not restricted to *C. perfringens* since several MLS-resistant isolates of both *C. difficile* and *C. paraputrificum* share sequence similarity with an *ermP* probe. In addition, the *ermP* gene hybridizes to, and shares restriction map identity with, the *ermB/ermAM* gene from the promiscuous *E. faecalis* plasmid pAMβ1 (11). In *C. difficile* at least one *ermP*-like erythromycin resistance determinant is located on a conjugative transposon which is chromosomally located and is capable of encoding the transfer of erythromycin resistance to other bacterial genera. The *C. difficile* erythromycin resistance gene, which was designated as *ermZ*, also hybridizes with the *ermB/ermAM* genes (66). Conjugative transfer of an MLS determinant from *Clostridium innocuum* to *C. perfringens*, in the absence of plasmid transfer has also been demonstrated (105).

DNA sequence analysis has shown that the sequence of the *ermP* gene is identical to the previously determined sequence (19, 120) of the *erm* gene from pAMβ1 and very similar to the other Erm B class MLS resistance determinants (12). On the basis of these data, and the designation of the *E. coli* erythromycin resistance determinant, which also belongs to the Erm B class, as the *ermBC* gene (20), it is proposed that the *C. perfringens ermP* gene be referred to as the *ermBP* gene (12) and that the *C. difficile ermZ* gene be known as the *ermBZ* gene.

Sequence analysis has shown that the constitutive *ermBP* gene is located between two 1.3-kb directly repeated sequences (Fig. 5). (10a, 164). The recombinant *ermBP* plasmid pJIR122, which contains both of the direct repeats, is unstable in *recA* strains of *E. coli*, with erythromycin-sensitive derivatives which lack one copy of the direct repeat being isolated at high frequency. As expected, plasmids containing one direct repeat are stable (11). The behavior of this determinant in *E. coli* is very similar to that of the *C. perfringens* transposons Tn*4451* and Tn*4452* (4). However, there is no evidence that the ErmBP determinant can transpose in either *C. perfringens* or *E. coli*.

The enterococcal plasmid pAMβ1 is a broad-host-range conjugative plasmid which has the ability to code for its own transfer to many different species of bacteria (74). Conjugative transfer to the clostridia has also been demonstrated (147, 155, 233). From these data it is reasonable to postulate that the *ermBP* gene arose from the transfer of a pAMβ1-like plasmid into *C. perfringens* followed by the subsequent transposition, or recombination, of the *erm* gene into the chromosome or a nonconjugative plasmid. Comparative sequence analysis of the *ermBP* gene region and the other

Erm B class determinants revealed that the identity between these resistance determinants extended beyond the respective *erm* genes. The identity between the pAMβ1 *erm* gene and *ermBP* extends into both of the *C. perfringens* direct repeats. However, pAMβ1 does not contain complete copies of both direct repeats. It therefore seems likely that the *C. perfringens* Erm BP determinant is more closely related to the primordial Erm B class determinant. It is suggested that the *C. perfringens* gene arose from a conjugative enterococcal or streptococcal plasmid which had complete copies of the direct repairs (10a).

GENETIC MANIPULATION OF *C. PERFRINGENS*

Transformation of *C. perfringens*

Genetic analysis in *C. perfringens* was limited for many years because there were no known mechanisms for genetic exchange. Early studies were therefore limited to the isolation and characterization of relevant mutants (36, 169, 171, 185, 191). Although conjugation mechanisms were discovered in the 1970s (18, 168, 184) and have been important in the analysis of *C. perfringens* R plasmids, they have not played a major role in the genetic analysis of chromosomal genes. The major advances in *C. perfringens* genetics have awaited the development of transformation methods.

Protoplast transformation. The first report of transformation in *C. perfringens* involved the polyethylene glycol-mediated transformation of protoplasts (70). L-phase variants of *C. perfringens* 11268 CDR (a derivative of the same strain which originally harbored pCW3) were generated by penicillin treatment in the presence of 0.4 M sucrose. The L-phase cells were transformed to tetracycline resistance by using the conjugative R plasmid pJU124. The transformation procedure involved the addition of the plasmid DNA to the protoplasts in the presence of high concentrations of polyethylene glycol. Transformants were obtained at low frequencies (ca. 1.4×10^{-6} transformant per μg of DNA per viable cell), but all of the transformed cells were still in the form of L-phase variants. Reversion to vegetative cells was not obtained. However, it was observed that autoplasts (protoplasts derived from autolysis) were able to be regenerated to produce rods with cell walls and could be transformed with *C. perfringens* plasmid DNA (70).

These methods were subsequently used to introduce shuttle plasmids into *C. perfringens* (195). L-phase variants could be transformed to tetracycline resistance with *E. coli*-derived plasmids but at frequencies some 2 orders of magnitude lower than those obtained with the same plasmid isolated from *C. perfringens*. Unfortunately, *C. perfringens* autoplasts could not be transformed with DNA which originated in *E. coli*, presumably because an outgrowth step in liquid broth was required (195). To obtain rod-shaped walled *C. perfringens* cells containing a plasmid originating in *E. coli*, a cumbersome two-step transformation procedure was required. This method involved the transformation of L-phase variants with plasmid DNA from *E. coli*, the extraction of plasmid DNA from the resultant transformants, the transformation of autoplasts with that DNA, and the regeneration of the autoplasts to rods (195). Although these studies made a very valuable contribution to the development of *C. perfringens* genetics and enabled the first generation of shuttle vectors to be constructed (195), the procedure was clearly too complex for routine use. Other laboratories had difficulty in obtaining the correct conditions for transformation, and the methods were not able to be used for the isolation of transformed rods. However, a micromethod based on this technique was developed for the transformation of other *C. perfringens* L forms (117) and successfully used for the construction of an additional shuttle plasmid (159).

Electroporation. The major advances in transformation methods resulted from the application of the technique of electroporation to *C. perfringens* (7, 182). Electroporation involves the application of a high-voltage electric field to vegetative bacterial cells for a very short period. The electric pulse creates pores in the bacterial cell membrane and allows the passive influx of DNA molecules (23). Initial studies involved the transformation of *C. perfringens* 3624A with the enterococcal plasmid pAMβ1 and the shuttle plasmid pHR106. Very small numbers of transformants (1.5×10^2 to 1.2×10^3 transformants per μg of DNA) were obtained (7). This brief report was followed by another study, from the same laboratory, in which extensive modifications of these conditions were reported (91). When the shuttle plasmid pAK201 was used, somewhat higher transformation frequencies (ca. 10^4 transformants per μg of DNA) were obtained. The authors commented that they routinely used late-stationary-phase cells and postulated that these cells may be more suitable for electroporation because they were partially autolyzed. No transformants were obtained when mid-log-phase cells were used (91). In another study, four type A strains and one type C strain were found to be transformable, but no transformants were obtained with the three type B strains tested (8). In that study some minor modifications of the original method (7) used by the workers in the Blaschek laboratory were reported (8), but, surprisingly, no mention was made of the alternative electroporation conditions reported from either their own (91) or other (182) laboratories, even though plasmids and strains from those studies were used. There is one other study in which electroporation was used to obtain *C. perfringens* transformants (148). In these experiments, a modification of the glycerol method previously reported (91) was found to yield optimal numbers of transformants (4.4×10^3 transformants per μg of DNA).

Previous workers, in their studies on polyethylene glycol-mediated transformation of the L-form strain L-13, commented that there was little difference in the transformation efficiencies of plasmid DNA derived from *E. coli* or *C. perfringens* (117, 159). These data could be readily explained if it was assumed that L-13 lacked a restriction and modification system. Derivatives of the vegetative strain 13, the parent of L-13, would therefore be excellent candidates for *C. perfringens* transformation recipients. A systematic study of the parameters affecting the electroporation-mediated transformation of strain 13 has been carried out, and the results have shown that it is an excellent transformation recipient (182). The effect of growth phase, DNA concentration, electric field strength, time constant, and cell density on the efficiency of transformation of strain 13 with pHR106 were all studied. The results showed that optimal numbers of transformants (ca. 3×10^5 transformants per μg of DNA) were obtained when the cells were harvested in early logarithmic growth phase and were treated at maximal field strength. The optimal cell density for efficient transformation was between 1×10^8 and 5×10^8 viable cells per ml. The efficiency of transformation (ca. 2.5×10^{-4} transformant per μg of DNA per viable cell) was high enough to allow the direct cloning of the *tet*(P) gene in *C. perfringens* (182). Unlike the results of other studies (7), no effects on cell viability were observed after electroporation treatment. Sig-

TABLE 5. *C. perfringens-E. coli* shuttle plasmids

Shuttle plasmid	Size (kb)	*C. perfringens*		*E. coli*		Reference
		Replicon	Selection[a]	Replicon	Selection[a]	
pJU12	11.6	pJU121	Tc	pBR322	Ap Tc	195
pJU13	12.2	pJU122	Tc	pBR322	Ap Tc	195
pJU16	12.2	pJU122	Tc	pBR322	Ap Tc	195
pHR106	7.9	pJU122	Cm	pSL100	Ap Cm	159
pAK201	8.0	pHB101	Cm	pBR322	Cm	91
pSB92A2	7.9	pCP1	Cm	pHG165	Ap Cm	148
pTG67	6.6	pIP404	Cm	pUC18	Ap Cm	46
pJIR418	7.4	pIP404	Em Cm	pUC18	Em Cm XG	191a

[a] Tc, Ap, Cm, and Em represent resistance to tetracycline, ampicillin, chloramphenicol, and erythromycin, respectively. XG represents screening for β-galactosidase production on X-Gal medium.

nificant strain dependence was found, as two other *C. perfringens* strains were refractory to transformation (182). Strain variation in transformation efficiency was later reported by other workers (8).

The initial electroporation transformation experiments with strain 13 were unsuccessful (182). Therefore, on the basis of results obtained with the lactic streptococci (151), the cells were pretreated with lysozyme prior to electroporation. No transformants were obtained. However, pretreatment with lysostaphin, a peptidase which cleaves the pentaglycine bridge in the *S. aureus* cell wall (21), yielded large numbers of transformants. Subsequent experiments showed that the optimal lysostaphin concentration was 2 to 20 μg/ml (182). Although the *C. perfringens* cell wall has been shown to contain glycine (178), it is not known whether the active factor involved is lysostaphin or an enzyme which is a contaminant of commercial lysostaphin preparations. It was assumed that lysostaphin partially digested the *C. perfringens* cell wall so that the plasmid DNA now had access to the pores subsequently created in the cell membrane by electroporation. This conclusion is in agreement with the suggestions made by previous workers regarding the potential role of autolysins (91).

This lysostaphin dependence is somewhat variable, and other workers have reported that they could obtain transformants of strain 13 without pretreatment with lysostaphin (159a). Subsequently, we have also, at times, obtained transformants without pretreatment. The reason for this variability is not known, but it is not due to changes in the quality of the DNA or in strain variation (181a). The most likely explanation is that use of different commercial batches of culture medium results in alterations in either the thickness or composition of the strain 13 cell wall. Lysostaphin pretreatment is still routinely carried out in our laboratories, and some *C. perfringens* strain, such as derivatives of CPN50, are transformed efficiently only under these conditions (47a).

In summary, it is clear that strain 13 offers the greatest potential for use as a *C. perfringens* transformation recipient. Strain 13 is currently the only *C. perfringens* strain that yields enough transformants to enable the direct cloning of genes in *C. perfringens*. Although other strains are transformable, the frequencies obtained are lower and their use will require optimization of the transformation conditions in each case. Some strains may not be transformable. Mobilization, by either pCW3 or pIP401, of plasmids introduced into strain 13 by electroporation should prove to be a useful means of introducing recombinant plasmids into these organisms.

C. perfringens-E. coli Shuttle Plasmids

The development of *C. perfringens* gene-cloning systems requires the construction of shuttle vectors capable of independent replication and selection in both *C. perfringens* and *E. coli*. The antibiotic resistance genes *tet*(P), *catP*, and more recently *ermBP* have proven invaluable for the construction of these vectors because they are all expressed in both *C. perfringens* and *E. coli*. A variety of *C. perfringens* replicons have been used, but we suggest that the most valuable shuttle plasmids will be those based on the very well characterized bacteriocin plasmid pIP404.

The first shuttle plasmids that were constructed used two *C. perfringens* cryptic plasmids (pJU121 and pJU122) and the *E. coli* plasmid pBR322 as replicons and the *tet*(P) genes from pCW3 and pJU124 (195). *E. coli* transformants were selected by resistance to ampicillin. The resultant plasmids (Table 5) were used to transform *C. perfringens* protoplasts, autoplasts, and L forms to tetracycline resistance (117, 159, 195).

The most commonly used shuttle plasmid has been pHR106 (159) (Table 5), which was the first vector to use the *C. perfringens catP* determinant from the recombinant plasmid pJIR62 (6). The plasmid pHR106 also uses the *C. perfringens* pJU122 replicon and has been used to transform both L forms (117, 159) and vegetative cells (7, 91, 182). The *catP* gene also has been used to construct pAK201, a shuttle vector based on the caseinase-encoding plasmid pHB101 (91), and pSB92A2, which incorporates the cryptic plasmid pCP1 (148).

All of the shuttle vectors reported in the literature (Table 5) can be used to transform *C. perfringens* to either tetracycline or chloramphenicol resistance. However, none of them are really suitable for use in pathogenesis studies that require the cloning and analysis of *C. perfringens* toxin genes. All of these vectors, with the exception of pAK201, contain an intact *E. coli* ampicillin resistance gene, *bla*. Since β-lactamase-mediated ampicillin resistance has not been found in *C. perfringens* (224), plasmids containing *bla* genes should be avoided in experiments involving the introduction of cloned *C. perfringens* toxin genes onto multicopy plasmids in *C. perfringens*. The plasmid pAK201 does not carry an intact *bla* gene but does have a complete copy of the caseinase, or λ-toxin, gene. This gene is not functional in pAK201, and the reasons why it is not expressed are not clear, because the gene has not been sequenced or otherwise characterized. Therefore, this vector plasmid should also be avoided in pathogenesis studies. Finally, all of the *C. perfringens* replicons used in the construction of the existing

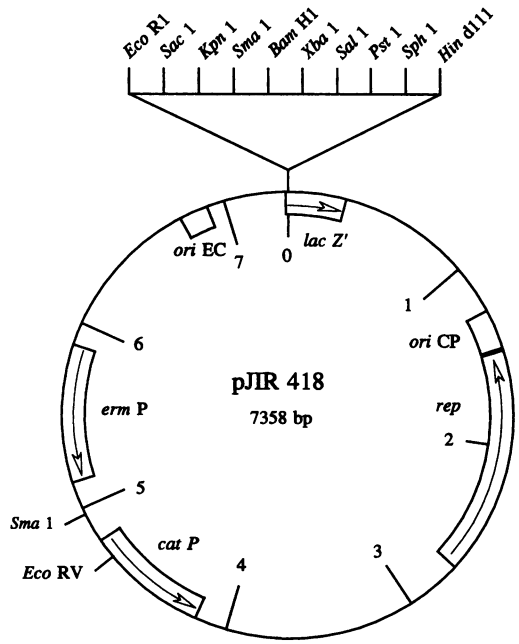

FIG. 6. Organization and genetic map of the *C. perfringens-E. coli* shuttle plasmid pJIR418. The location and direction of transcription of the *ermBP* gene from pIP402 (11), the *catP* gene from pIP401 (6), the *rep* gene from pIP404 (45), and the *lacZ'* gene from pUC18 (230) are indicated. The plasmid pJIR418 (191a) contains the multiple cloning region from pUC18 (at position 0) and both *E. coli* (*oriEC* from pUC18) and *C. perfringens* (*oriCP* from pIP404) origins of plasmid replication.

vectors are uncharacterized plasmids which have not been sequenced and whose mechanisms of replication are unknown. It seems obvious that the next generation of *C. perfringens-E. coli* shuttle vectors should use the completely sequenced and well-characterized plasmid pIP404. Since this plasmid does not replicate by a rolling-circle mechanism involving a single-stranded intermediate (45, 46), derivatives of pIP404 should be stable in *C. perfringens* (see other sections of this review).

A new shuttle plasmid, pJIR418, has been constructed in one of our laboratories (Fig. 6) (191a). This plasmid contains the *C. perfringens catP* and *ermBP* genes but does not have an intact *bla* gene. It contains the replication region, *lacZ'* gene, and multiple cloning site from pUC18 and the replication region from pIP404. Selection in both *C. perfringens* and *E. coli* relies on either chloramphenicol or erythromycin resistance. The normal 6-base recognition sites in the multiple cloning region can be used for cloning in *E. coli*, with screening for recombinants on 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) medium. This property is absent from all other *C. perfringens-E. coli* shuttle plasmids. In addition, blunt-end cloning into the *Eco*RV site located in the *catP* gene can be used to insertionally inactivate the chloramphenicol resistance determinant, which allows for direct screening of recombinants in *C. perfringens* after selection for erythromycin resistance. The vector uses the pIP404 replicon, which, as outlined elsewhere in this review, has replication properties that are ideal for use as a cloning vector. The final important feature of pJIR418 is that the entire sequence of this plasmid is known because the sequence of each of its component parts was already available. The detailed molecular analysis of recombinant clones constructed in pJIR418 will therefore be greatly facilitated.

To test the usefulness of pJIR418, we have cloned the *C. perfringens* phospholipase C gene, *plc*, from the *E. coli* recombinant plasmid pTox6 (176) into pJIR418 and successfully reintroduced the resultant recombinant plasmid into *C. perfringens* strain 13 (191a). The success of this relatively simple manipulation augurs well for the use of pJIR418 in studies on the pathogenesis of *C. perfringens* infections. In particular, it should be possible to use pJIR418, in combination with the electroporation-mediated transformation of strain 13, for the direct cloning of *C. perfringens* virulence genes whose gene products are too large to be successfully expressed in *E. coli*.

Transposon Mutagenesis of *C. perfringens*

Molecular approaches to the study of bacterial pathogenesis generally involve the isolation of mutants defective in the production of specific virulence factors, the reintroduction of functional virulence genes into these mutants, and the testing of these strains in animal or tissue culture models. Transposon mutagenesis is site specific and is therefore one of the preferred methods for the isolation of such mutants because it is relatively easy to ensure that the mutation is in the correct gene and that only the gene being studied has been altered. Although two transposons, Tn*4451* and Tn*4452*, have been characterized from *C. perfringens* (4), the conditions for transposition in *C. perfringens* have not been elucidated; hence, these transposons are currently of limited value as genetic tools. The best candidates for transposon mutagenesis are therefore the *E. faecalis* transposons Tn*916* (50, 181) and Tn*917* (232), both of which have been used for transposon mutagenesis in other gram-positive bacteria (10). To the best of our knowledge, there have been no reports of the use of Tn*917* in *C. perfringens*, although the *ermB* gene carried on this transposon should be expressed in *C. perfringens*.

In contrast, the conjugative transposon Tn*916*, which contains a *tet*(M) gene, has been successfully introduced into *C. perfringens* (6a, 181a, 196a). Transfer of Tn*916* from *E. faecalis* to an erythromycin-resistant derivative of *C. perfringens* 13 has been achieved by filter mating and selection for tetracycline and erythromycin resistance. Transconjugants were detected at a frequency of approximately 10^{-7} to 10^{-8} per viable recipient. Hybridization analysis confirmed that the *C. perfringens* transconjugants carried a chromosomal copy of Tn*916*. Only three of the 20 transconjugants examined carried more than one copy of Tn*916* (196a). Transconjugants obtained in these matings have been used successfully as donors in further *C. perfringens-C. perfringens* matings to demonstrate the feasibility of carrying out conjugative Tn*916* mutagenesis directly in *C. perfringens* (181a, 196a).

Other workers have used an alternative, equally effective approach to Tn*916* mutagenesis in *C. perfringens* (6a). Tn*916* was introduced by transformation in pAM120, an *E. coli* recombinant plasmid which carries Tn*916* but does not replicate in *C. perfringens*. Hybridization analysis showed that Tn*916* had been randomly incorporated into the *C. perfringens* chromosome. It was noted that multiple Tn*916* insertion events occurred at high frequency. All three of the research groups to have studied Tn*916* in *C. perfringens* have observed multiple insertion events (6a, 181a, 196a). It will be essential to show, by screening large numbers of insertion derivatives, that the incidence of multiple inser-

tions is within workable limits. If so, Tn*916* mutagenesis should prove to be invaluable for the analysis of genes coding for toxins and other *C. perfringens* virulence factors. Otherwise it may be very worthwhile investigating the potential of mutagenesis with Tn*917*.

GENETICS AND CHROMOSOME MAPPING

Classical Genetics and the Advent of Cloning

C. perfringens is a natural auxotroph and requires exogenous supplies of 11 amino acids and several vitamins for growth (17, 185). Although this is consistent with its saprophytic lifestyle and its role in the putrefaction process, it has complicated the classic genetic approach, namely the characterization of mutants with defined biosynthetic lesions. Early genetic studies, involving chemical mutagenesis, led to the isolation of mutants which required uracil and lysine as well as to the definition of a minimal medium suitable for the isolation of further mutants with defects in additional metabolic pathways (185). A similar approach was used to identify mutations which affected the formation of spores (36). Attempts to isolate transducing phages or plasmids capable of chromosome mobilization were unsuccessful (18), and progress in *C. perfringens* genetics was slow until the introduction of recombinant DNA technology.

Among the early DNA fragments cloned were various components of resistance and bacteriocin plasmids, as described in the preceding sections. The first successful cloning of a gene from the chromosome of *C. perfringens* was reported by Fairweather et al. (40), who isolated a hemolytic transducing phage from a lambda library and mistakenly identified it as a clone producing the θ-toxin. Subsequent studies clearly attributed the hemolysis to the activity of the multifunctional α-toxin, described below. Since that time, more than 30 known genes have been cloned or identified by cross-hybridization with heterologous probes, and gene banks have been established in phage, cosmid, and plasmid vectors.

Chromosome Mapping

Pulsed-field gradient gel electrophoresis of macro-restriction fragments has had a major impact on *C. perfringens* research and has led to the establishment of detailed physical and genetic maps of seven strains. Construction of restriction maps was facilitated considerably by the high dA+dT content (75%) of the genome. Canard and Cole (24) reasoned that the use of six restriction enzymes with dG+dC-rich recognition sequences would generate a detailed physical map with arbitrary genetic intervals (50 to 100 kb on average) suitable for the localization of cloned genes, assuming that the sites were distributed randomly on a typical bacterial chromosome (i.e., about 4 Mb in size). After many endonucleases had been tested, the six enzymes retained for use were *Apa*I, *Fsp*I, *Mlu*I, *Nru*I, *Sac*II, and *Sma*I; of these, *Mlu*I proved to be particularly valuable as it cleaved only five times in the genome of the type A strain CPN50. This meant that it was possible to isolate *Mlu*I end probes, and linking clones which spanned the *Mlu*I site, for use in hybridization mapping analysis. The end probes were used to produce maps of individual *Mlu*I fragments that had been partially digested with the other five enzymes, and these fragments could then be attached to the following *Mlu*I fragment by means of the linking clones. In this way, it was established that CPN50 possesses a single circular chromosome of ca. 3.6 Mb on which more than 100 restriction sites have been situated. On inspection of the map, it was clear that the distribution of the sites was nonrandom and that certain areas were prone to multiple cleavage; these areas were later shown to correspond to the rRNA operons (43). Nonetheless, the initial objective was achieved, and 50 physical intervals, varying from less than 5 to 250 kb, were defined on the chromosome. By means of DNA hybridization, the positions of all the cloned *C. perfringens* genes, as well as those of housekeeping genes of conserved sequence, were identified (24). The current gene map is shown in Fig. 7, superimposed on the most recent restriction map of the chromosome of *C. perfringens* CPN50.

Subsequently, this study was extended to include three more type A strains (two from culture collections and the other isolated from a recent case of gangrene) in addition to a well-characterized member of the other serotypes, B, C, D, and E (25b, 30). The objective was to see whether polymorphic genomic regions could be correlated with differences in pathogenesis and host specificity. Detailed physical maps were generated by the combined indirect end labeling and linking approach outlined above for the *Mlu*I fragments of all strains, except for the type C isolate, which produced copious amounts of a highly stable DNase. Comparison of the maps revealed a remarkably constant chromosome organization with highly conserved restriction sites, fragment sizes, and genetic loci. Comparative genome mapping showed the *C. perfringens* isolates to comprise a homogeneous group in which the type A, B, D, and E strains are closely related. The positions of the rare variant regions are indicated in Fig. 7; in most cases these are due to small insertions and deletions and not to gross chromosome rearrangements such as inversions. The largest insertions detected, which were ca. 50 kb in size, probably correspond to integrated prophages; experimental confirmation of this has been obtained in several cases (25). In contrast, although the smallest insertions, 1 to 2 kb, may be indicative of the presence of insertion sequences or transposons, no evidence is available to support this suggestion. Clearly, it is tempting to attribute novel virulence functions to some of these insertions, but, owing to the lack of appropriate probes, no direct proof is available. It should be possible, however, to clone the corresponding regions by using a subtractive hybridization approach and to determine whether they encode potential factors of virulence. Alternatively, it is quite conceivable that the genes coding for toxins not found in type A strains are borne by plasmids or episomes; this will be discussed further below.

Housekeeping Genes

All of the strains of *C. perfringens* examined appear to contain 10 rRNA operons, and this undoubtedly contributes to the fast growth of the organism (24, 43). One rRNA operon, *rrnA*, is linked to the DNA gyrase genes, *gyrA* and *gyrB*, and in the related organisms *B. subtilis* and *S. aureus* this arrangement is associated with the chromosomal origin of replication, *oriC* (71, 73). By analogy, it is quite probable that *rrnA* defines the origin of replication of *C. perfringens*, and, in this case, seven of the *rrn* operons are located on the clockwise side of the origin and the remaining three on the counterclockwise side (Fig. 7).

Interspersed with the rRNA operons are many tRNA genes, and these two gene families are confined to an area, spanning the putative origin of replication, which represents a mere 30% of the chromosome (24). This compact arrange-

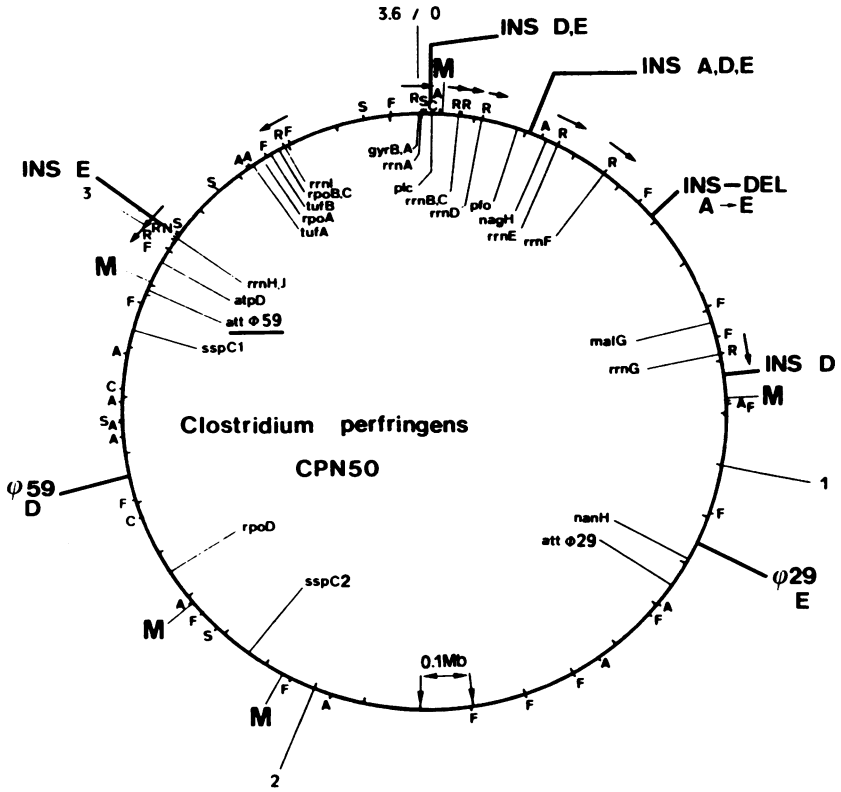

FIG. 7. Physical and genetic map of the chromosome of *C. perfringens* strains. The map is based on *C. perfringens* CPN50 (24). It also incorporates a summary of comparative genome-mapping studies on type A, B, D, and E isolates of *C. perfringens*. Positions of insertions (INS) and deletions (DEL) are indicated on the map of the paradigm strain CPN50 (24) and are identified by the corresponding toxin type. Note the different locations of the *att* sites for Φ29 and Φ59 in *C. perfringens* types A, D, and E. The positions of all of the genes indicated have not been verified experimentally but are likely to be as shown, because no differences in the order or approximate location of the 20 markers tested were seen in the seven strains examined (25b). Restriction sites for *Apa*I (A), *Fsp*I (F), *Mlu*I (M), *Sac*II (C), and *Sma*I (S) are positioned on the outside of the circle; R denotes the rare site cluster, *Sac*II (twice), *Sma*I (twice), *Nru*I, *Sma*I, and *Nru*I, which occurs in each RNA operon. The scale in 0.1-Mb intervals is shown on the inside, as are known genes or genetic loci identified by cross-hybridization with heterologous probes. Known clostridial genes and their products are as follows: *gyrA*, DNA gyrase; *malG*, unidentified gene showing homology to *malG* of *E. coli* (71a); *nagH*, β-*N*-acetylglucosaminidase, *nanH*, sialidase; *pfo*, perfringolysin or θ-toxin; *plc*, phospholipase C or α-toxin; *rrnA* to *rrnI*, rRNA operons; *sspC1* an *sspC2*, small acid-soluble proteins; *tufAB*, elongation factor Tu; *att*Φ29, integration site for Φ29; *att*Φ59, integration site for Φ59. Other genetic loci are as follows: *atpD*, β subunit of ATP synthase; *gyrB*, DNA gyrase; *rpoA*, α subunit of RNA polymerase; *rpoB*, β subunit of RNA polymerase; *rpoC*, β′ subunit of RNA polymerase; *rpo*D, σ^{43} subunit of RNA polymerase.

ment is highly beneficial to a sporulating bacterium such as *C. perfringens* as it ensures an immediate supply of components for the translational apparatus following spore germination, with the corresponding genes being among the first to be replicated from the bidirectionally active *oriC*.

Transcription of the rRNA operons proceeds from powerful promoters, described below (43), that are recognized by RNA polymerase containing the vegetative σ factor. All of the genes encoding RNA polymerase subunits have been identified on the chromosome (24), as have the coding sequences for elongation factor Tu and ATP synthase. Although sporulation was the first developmental process of *C. perfringens* to be studied genetically, little is known about the loci involved, although recently the genes for two spore components, the small acid-soluble spore proteins, were cloned and sequenced (15, 56, 72) and positioned on the chromosome map (Fig. 7). This analysis showed that the products of the *sspC1* and *sspC2* genes, which account for 20% of the protein content of the spore, share 90% sequence identity and approximately 40% identity with small acid-soluble spore proteins α and β, from *Clostridium bifermentans* (22).

Transcriptional Signals

The major form of RNA polymerase has been purified from exponentially growing *C. perfringens* and shown to contain two polypeptides of 145 and 135 kDa, corresponding to the β and β′ subunits, and three smaller proteins of 52, 48, and 44 kDa, which may represent the α subunit and various σ factors (47). As one might expect, the enzyme preferentially employs dA+dT-rich promoters over their dG+dC-rich counterparts in an in vitro transcription system and has been a useful tool in molecular genetic studies. All of the biochemically characterized promoters from *C. perfringens* are shown in Fig. 2. These promoters fall into two groups, those that are constitutively expressed, or transcribed by the major form of RNA polymerase (Fig. 2b), and those which are activated following DNA damage (Fig. 2a).

Owing to the high dA+dT content, visual inspection of DNA sequences is a hazardous method of spotting *C. perfringens* promoters, and even when these have been defined experimentally the −35 and −10 regions sometimes show rather poor resemblance to the extended consensus promoter sequences proposed for gram-positive bacteria

(63). It may be significant, in an organism with a bias against dC and dG, that the frequency of guanosine residues throughout the promoter region, but especially in the 10 bp preceding the −35 hexamer, is strikingly high. Likewise, the top strand of promoter regions is virtually free from cytosine residues (Fig. 2b). Together with the classic sequence elements, these features possibly contribute to promoter recognition by RNA polymerase. On the basis of the sample of promoter sequences available, a consensus *C. perfringens* promoter sequence has been deduced (Fig. 2). This agrees well with the extended promoter consensus sequence proposed by Graves and Rabinowitz (63), although the −45 region is less well conserved and the T-TG motif preceding the −10 region is most prominent.

The regulatory regions for four of the rRNA operons have been studied in detail, and in three cases transcription occurs from tandem promoters, as has been found in other bacteria (43). The proximal promoters contain canonical TTGACA sequences in the −35 region, whereas the distal promoters possess no clearly defined −35 sequences. In all cases the −10 hexamers are preceded by one nucleotide by TG motifs which are known to compensate for atypical −35 sequences (89, 150). Transcription of *rrnH* is from a single promoter which bears closer resemblance to the distal promoters of the other operons. All of the *rrn* control regions contain sequence motifs which are very similar to those known to mediate the stringent response in *B. subtilis* (43), and it is therefore likely that a similar global control system exists in *C. perfringens*.

Among the first promoters to be characterized were those controlling two UV-inducible transcriptional units carried by the bacteriocinogenic plasmid pIP404 (47, 48). Although one of these five promoters (P4) was weakly constitutive (Fig. 2b), the others were activated only after DNA damage. Three of the promoters are strikingly similar and have the same −35 and −10 regions in TTTACA and CTTTTTAT, respectively. As none of these promoters is recognized by the major form of RNA polymerase, this was taken as evidence that the SOS system is positively controlled in *C. perfringens*, unlike the situation in members of the family *Enterobacteriaceae*, in which the LexA repressor shuts off expression under normal physiological conditions (221).

As *C. perfringens* is a sporulating bacillus, it is most probable that the genes required for the different stages of spore formation will be the targets of a complex regulatory network involving a host of different σ factors and activator molecules, as found in *B. subtilis* (205). To date, the only sporulation genes which have been cloned are the *ssp* genes, and indirect evidence suggests that these should be transcribed from forespore-specific promoters by RNA polymerase containing a σ factor analogous to σ^G (72, 141). It is anticipated that as more genes are cloned and characterized, a wealth of detail about their various control systems will emerge.

Translational Signals

The translation initiation sites preceding *C. perfringens* coding sequences all contain a ribosome-binding motif showing reasonable complementarity to the 3′ end of the 16S rRNA (AAGGAGGA) (43). This site is invariably situated 6 to 10 bases upstream of the initiation codon, which, in all but one of the cases thus far characterized, is AUG. The exception is a GUG initiation codon used by a poorly expressed gene, *rep*, of pIP404 (45). By far the most striking feature of the mRNAs is their extraordinarily biased codon usage, which is a reflection of the extremely high dA+dT content of the *C. perfringens* chromosome. The average codon usage for 21 genes of chromosomal and plasmid origin is presented in Table 6 and compared with the corresponding data from *E. coli* and *B. subtilis*. Codons composed of two or more G · C base pairs have been selectively excluded from *C. perfringens* genes by unknown evolutionary pressures, and 7 of the 61 sense codons are virtually never used. For example, the major codons for Arg in *E. coli*, CGU and CGC, are rarely found in *C. perfringens* mRNAs in which AGA is predominant (143). This codon is rigorously avoided by *E. coli*, and the availability of the cognate tRNA has been identified as a rate-limiting factor for expression in this organism. Other examples of codons for which the frequency of utilization varies between 1 and 2 orders of magnitude between *E. coli* and *C. perfringens* are UUA (Leu), AGU (Ser), ACA (Thr), GGA (Gly), and AUA (Ile). It is striking that the codon usage in antibiotic resistance genes isolated from *C. perfringens* (*catP*, *catQ*, and *ermBP*) is significantly less biased than that of chromosomal genes (data not shown), suggesting that horizontal transfer may have occurred.

To date, no *E. coli* genes have been shown to be expressed in *C. perfringens*, although many *C. perfringens* coding sequences have been cloned in the gram-negative host. The diametrically opposed codon usage is likely to represent a barrier to expression, and to assess this possibility we have analyzed a number of *C. perfringens* genes for their rare codon content, their context, and the degree of expression observed. In most cases the T7 expression system (Φ10 promoter and endogenous T7 RNA polymerase) was used to drive transcription in *E. coli* (206) and the amount of protein synthesis was determined in vitro (by labeling with [^{35}S]methionine) or in vivo (Coomassie blue-stained proteins). As can be seen in Table 7, the number of rare codons did not limit expression until the genes became very large. At this point no full-length protein was produced in vitro, although peptides corresponding to translational intermediates could be detected (31a). Similar results have been obtained for expression of other large clostridial genes such as those encoding the tetanus toxin (39) or the *C. difficile* cytotoxin A (87, 220). These findings are difficult to reconcile with existing models for the limitation of gene expression in *E. coli* by rare codons, such as those proposed by Chen and Inouye (26), who found a correlation between expression levels and the frequency of rare codons in the first 30 codons, or Varenne and Lazdunski (219), who showed that clusters of three or more rare codons slowed or stopped translation. In the genes studied there is no correlation between rare-codon content, position, and context. The gene length is the decisive factor (Table 7). The longer the mRNA, the greater will be the probability that a ribosome will stall at a rare codon. Presumably the A+T-rich mRNA then succumbs to an exonucleolytic attack by RNases before translation has the chance to recommence. These observations should be borne in mind if one is considering expression cloning of large *C. perfringens* genes in *E. coli*, as the chances of success are clearly much greater if the constructs are shuttled back into *C. perfringens* prior to screening.

TOXINS AND VIRULENCE FACTORS

As one might expect of an organism responsible for such a variety of pathological conditions, ranging from relatively mild food poisoning to life-endangering myonecrosis, *C. perfringens* produces at least 16 potential virulence factors

TABLE 6. Codon usage in *C. perfringens* genes and comparison with *E. coli* and *B. subtilis*[a]

Amino acid	Codon	Usage (%) in:		
		C. perfringens	*E. coli*	*B. subtilis*
Arg	CGU	9.3	58.1	25.2
	CGC	1.5	35.0	17.5
	CGA	2.1	2.3	9.1
	CGG	1.5	3.2	11.1
	AGA	72.2	1.2	27.7
	AGG	13.4	0.3	9.4
Leu	CUU	14.4	8.6	26.1
	CUC	0.1	6.6	9.8
	CUA	9.2	1.8	6.3
	CUG	3.0	69.1	21.8
	UUA	66.5	5.8	22.4
	UUG	6.8	8.2	13.6
Ser	UCU	21.5	26.5	24.4
	UCC	3.4	25.6	12.0
	UCA	33.0	8.3	18.7
	UCG	0.6	11.4	10.0
	AGU	33.3	6.5	10.7
	AGC	8.2	21.6	24.1
Thr	ACU	41.8	23.8	14.8
	ACC	6.6	50.6	14.1
	ACA	47.0	5.9	43.3
	ACG	4.6	19.7	27.9
Pro	CCU	38.1	9.0	33.6
	CCC	1.1	6.0	9.8
	CCA	57.4	19.9	19.1
	CCG	3.4	65.1	37.5
Ala	GCU	39.9	27.9	27.5
	GCC	8.1	18.8	20.0
	GCA	48.3	22.9	27.1
	GCG	3.7	30.5	25.4
Gly	GGU	27.4	47.8	25.4
	GGC	5.2	40.8	29.6
	GGA	59.6	4.6	31.5
	GGG	7.8	6.8	13.5
Val	GUU	48.2	37.5	31.4
	GUC	3.0	12.9	24.7
	GUA	44.9	22.9	24.5
	GUG	3.9	26.8	19.4
Ile	AUU	39.9	37.3	50
	AUC	5.2	62.2	39.4
	AUA	54.9	0.5	10.6
Lys	AAA	73.8	76.7	75.4
	AAG	26.2	23.3	24.6
Asn	AAU	79.8	24.2	53.1
	AAC	20.2	75.8	46.9
Gln	CAA	85.4	26.6	54.2
	CAG	14.6	73.4	45.8
His	CAU	75.6	38.9	68.6
	CAC	24.4	61.1	31.4
Glu	GAA	78.9	73.4	69.5
	GAG	21.1	26.6	30.5
Asp	GAU	87.4	51.0	63.8
	GAC	12.6	49.0	36.2
Tyr	UAU	87.3	40.6	61.8
	UAC	12.7	59.4	38.2
Cys	UGU	69.8	42.0	45.7
	UGC	30.2	58.0	54.3
Phe	UUU	76.7	43.5	64.0
	UUC	23.3	56.5	36.0
Met	AUG	100	100	100
Trp	UGG	100	100	100

[a] Data for *C. perfringens* are compiled from the sequences of 21 known genes, of chromosomal or plasmid origin, comprising 6,239 codons. Data for *E. coli* and *B. subtilis* are from Ogasawara (143). Sources of the *C. perfringens* codons were references 9, 25a, 45, 72, 120, 176, 196, 215, and 218.

(124, 125, 194), including 12 toxins (α to ν), enterotoxin, non-α-δ-θ hemolysin, neuraminidase (sialidase), and the vigorous metabolic activity of the organism. Many of these virulence factors are simple hydrolytic enzymes secreted by the organism as part of its saprophytic life style in the soil, where they are probably involved in the putrefaction process. Their role in pathogenesis is therefore, at best, purely fortuitous. Only a selection of these factors are produced by a given isolate of *C. perfringens*, and the identity of some may well be rather dubious. It is most probable that more factors of pathogenicity will be discovered as advances are made in the detailed genetic analysis of *C. perfringens* pathogenesis.

In the last few years there has been considerable progress in our understanding of the genetics, molecular biology, and activity of some of the major pathogenic determinants, and these factors will now be addressed individually. Gene cloning and expression in *E. coli* has been of great value to studies of the mode of action, as it has allowed toxin preparations to be obtained free from contaminating activities and has enabled structure-function relationships to be elucidated.

TABLE 7. Correlation between rare codons, gene size, and expression in *E. coli*[a]

Gene	% Rare codons	Total no. of codons	Expression	
			In vitro	In vivo
catP	9.9	212	+	+
orfF	23.7	279	+	NT[b]
nanH	18.3	382	+	+
plc	14.3	398	+	+
pfo	16.2	499	+	+
bcn	22.1	890	−	−
nagH	17.5	943	−	−
tet	17.8	1,315	−	−

[a] Data taken from the literature and our unpublished observations: *catP* (196), *orfF* and *nagH* (25a), *nanH* (161), *plc* (175a, 176), *pfo* (214), *bcn* (44), *tet*, tetanus toxin (39).
[b] NT, not tested.

α-Toxin or Phospholipase C

Numerous observations suggest that the most important virulence factor produced by clostridial myonecrosis (gas gangrene)-causing isolates of *C. perfringens* is the α-toxin, the first toxin for which an enzymatic activity, phospholipase C, was demonstrated (103). The α-toxin is a truly multifunctional metalloenzyme and possesses both phospholipase C and sphingomyelinase activities, which are responsible for the cytotoxicity, necrosis, and hemolysis observed (133). The 50% lethal dose for mice is only 0.75 hemolytic units, and among the resulting symptoms are induction of inflammatory responses, smooth muscle contraction, platelet aggregation, shock, bradycardia, and greatly reduced myocardial function culminating in death (201).

Cloning, mapping, and expression of *plc*. In 1989 the successful cloning of the α-toxin gene, *plc*, was reported by no less than five different research groups. Four of these groups cloned the gene from the α-toxin-overproducing strain NCTC 8237 (95, 145, 208, 213), whereas the fifth used a derivative of the heat-resistant strain NCTC 8798 (176). The various DNA sequences were virtually identical, and genes homologous to *plc* have been detected in several other clostridial species (210). The gene from *C. bifermentans* has also been cloned and characterized (213).

In all five toxigenic types (A to E) of *C. perfringens*, the *plc* gene is situated at the same locus, near the putative origin of replication and between two rRNA operons (25b). This location may well be of importance as this region of the chromosome is not only the first to be replicated but is also probably the best-conserved segment of the genome, as it contains many housekeeping genes.

plc is expressed from a constitutive promoter (Fig. 2b), which is also recognized by *E. coli* RNA polymerase (176). There are reports that growth medium composition affects the production of α-toxin (138, 139), suggesting the existence of a possible control network which responds to the availability of a given nutrient. Many bacterial virulence factor genes are controlled as part of global regulons (130), and some phospholipase C genes are induced in response to phosphate limitation (152). Phosphate does not appear to control *plc* expression (31b, 139), and further work is required to identify possible environmental regulatory signals. It is conceivable that the variable levels of α-toxin observed in different media are attributed to differences in the concentration of Zn^{2+} and Ca^{2+}, which are essential cofactors. In contrast, expression of four toxin genes (θ, κ, λ, and neuraminidase) is believed to be controlled by a single regulatory locus (77), which may correspond to the *pfoR* gene (189).

Structure-function relationships. Reasonable levels of α-toxin production have been obtained from the cloned *plc* gene in *E. coli*, where the enzyme is correctly processed and secreted to the periplasm (95, 145, 208). Purified α-toxin from this host has biochemical properties virtually indistinguishable from those obtained with toxin from *C. perfringens*, except for a difference in its pI (5.6 in *E. coli*; 5.48 in *C. perfringens*). One major advantage of using recombinant α-toxin in biological studies is the complete absence of the contaminating clostridial proteins which have plagued *C. perfringens* toxin research (134, 135). In this way it was demonstrated unambiguously (176) that the *plc* gene product possessed both the phospholipase C and sphingomyelinase activities which had previously been thought to be associated with α-toxin (136).

The availability of the primary structure of α-toxin, deduced from the nucleotide sequence of the *plc* gene, led to a major breakthrough in our understanding of its functional organization. Comparison of the amino acid sequences of the 370-residue mature form of α-toxin and the 245-residue phospholipase C from *Bacillus cereus* revealed extensive homologies and the presence of 65 identical equivalanced amino acid residues. In Fig. 8 the amino acid sequences of two α-toxins from different strains of *C. perfringens* are compared with the sequences of two phospholipase C enzymes from different isolates of *B. cereus* (52, 85). The crystal structure of one of the *B. cereus* enzymes, PC-PLC, has been deduced at a resolution of 0.16 nm (75), and this has been of great value in interpreting the organization of the clostridial α-toxin protein.

The α-toxin is clearly composed of a complete PC-PLC module fused to a COOH-terminal domain of 120 amino acid residues. It is apparent that all of the amino acids involved in coordinating the three zinc atoms found at the active site of PC-PLC (75) have obvious counterparts in α-toxin (Fig. 8). These include five histidine residues, and experimental evidence showing the importance of histidinyl groups for α-toxin activity has been obtained (209).

The crystallographic structure of PC-PLC (75) shows the enzyme to comprise 10 α-helical segments linked by β-turns of variable length. Structural predictions (176) suggest a very similar organization for α-toxin, with the additional COOH-terminal domain predicted to contain a further four α-helices. A number of observations indicated that this supplementary domain could contribute to the sphingomyelinase activity of α-toxin, but, before discussing them, it is important to summarize the situation in *B. cereus*. In addition to PC-PLC, *B. cereus* produces a sphingomyelinase (85, 229), and the two enzymes act synergistically as a hemolysin (52). The genes for both enzymes, *cerA* and *cerB*, have been cloned and sequenced and appear to make up an operon in which the PC-PLC gene, *cerA*, is the promoter-proximal cistron. In *C. perfringens* both enzyme activities are united in a single hemolytic toxin, suggesting that the present-day *plc* gene is the product of a distant gene fusion event. If so, the sequence which originated from *cerB* has diverged considerably more than the *cerA* module, as the 120-residue COOH-terminal domain of α-toxin shares no significant amino acid sequence homology with the 304-residue sphingomyelinase from *B. cereus*. However, predictive studies suggest that both polypeptides have a predominantly α-helical secondary structure and similar amphiphilic profiles (175a).

To test the hypothesis for a two-domain organization of α-toxin, two studies were performed independently. Titball and coworkers (208a) constructed a truncated form of *plc*, which encoded the domain homologous to PC-PLC, and characterized the biological activities of its product. As expected, the 249-residue protein retained phospholipase C activity but was unable to hydrolyze sphingomyelin or provoke hemolysis. Furthermore, the truncated protein exhibited greatly reduced toxicity. Some of these results were confirmed by workers in one of our laboratories (175b), who constructed a series of gene fusions between *plc* and the genes encoding *E. coli* alkaline phosphatase or β-lactamase. Hybrid proteins consisting of the 354 NH_2-terminal residues of α-toxin fused to the reporter enzymes were inactive in hemolysis or sphingomyelin hydrolysis and displayed greatly reduced phospholipase activity. All the available evidence indicates that α-toxin does indeed have a two-domain organization. However, it is not yet clear whether the protein possesses two distinct active sites, one in each domain, or

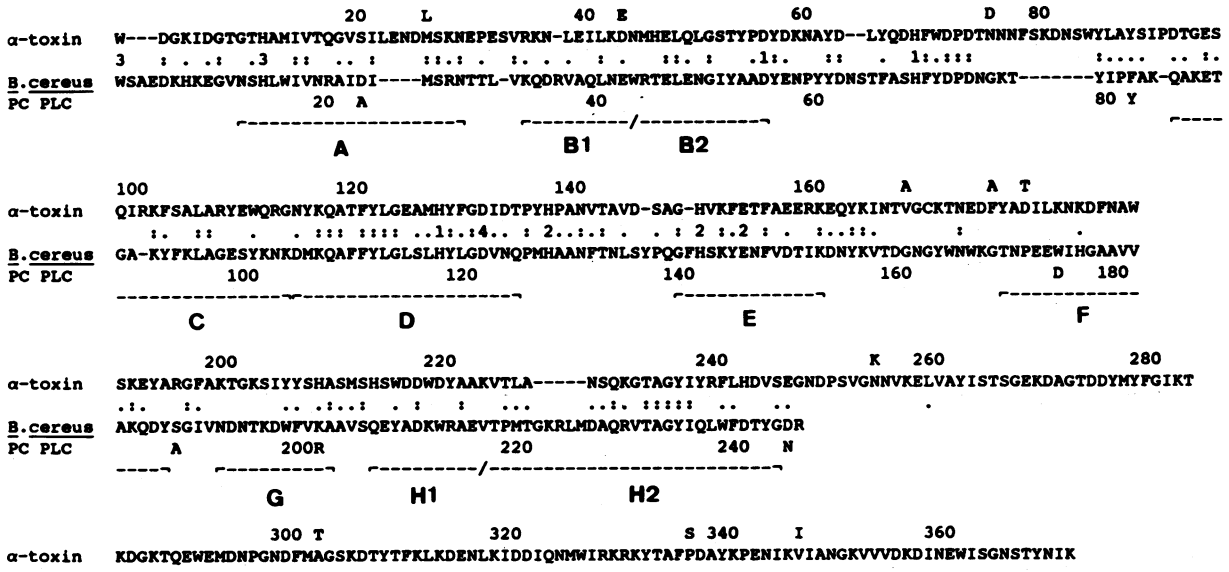

FIG. 8. Alignment of phospholipase C sequences. The primary structures of the α-toxin from *C. perfringens* NCTC 8798 and NCTC 8237 are aligned with the PC-PLC phospholipases from *B. cereus* ATCC 10987 and GP-4 (52, 85, 95, 145, 176, 208, 213). Gaps have been kept to a minimum, and only the residues of the enzymes from *C. perfringens* NCTC 8237 and *B. cereus* GP-4 which differ from their counterparts in NCTC 8798 and ATCC 10987 are shown. The positions of the α-helices (A to H_2) in PC-PLC established in structural studies (75) are shown. Residues known to be involved in coordinating the three zinc atoms in PC-PLC, and their counterparts in α-toxin, are highlighted; the numbers 1, 2, and 3 refer to the individual zinc atoms contacted, while 4 indicates an aspartic acid residue which coordinates zinc atoms 1 and 3. Symbols: :, identical residues; ., conservative substitutions.

whether there is a single active site, with broad substrate specificity, in the NH_2-terminal segment. If the latter possibility is correct, the COOH terminus must play a role in modulating enzyme activity. Elucidation of the crystal structure of α-toxin should clarify the situation.

β-Toxin

C. perfringens types B and C produce β-toxin, which is both lethal and necrotic (Table 1). Although type B strains cause concern in veterinary medicine, type C isolates can be important in human disease and are responsible for necrotic enteritis, which has decimated poorly nourished individuals in postwar Germany and New Guinea, where the disease is known as darmbrand and pigbel, respectively. The β-toxin has been purified to homogeneity and shown to correspond to a heat-sensitive 28-kDa protein which is highly sensitive to trypsin (88). This observation is of physiological significance since in normal individuals the toxin is rapidly inactivated by trypsin in the small intestine. Clearly, in times of starvation, trypsin levels are low and the levels of active β-toxin in the gut are therefore much higher. It was discovered that sweet potatoes, a vegetable staple often served as an accompaniment to pork (the usual source of contamination), contain a potent trypsin inhibitor, which may contribute to the incidence of pigbel (55, 140). Although there are unconfirmed reports that β-toxin production may be associated with a plasmid (35), very little genetic information is available.

ε-Toxin

Like the α-, β-, and ι-toxins, the ε-toxin is both lethal and necrotizing, although its precise biological activity has not yet been identified. It is produced by type B and D strains (Table 1) and is of great veterinary interest because it causes a rapidly fatal enterotoxemia amongst ungulates which is commonly referred to as pulpy kidney or overeating disease (125). The ε-toxin is produced in precursor form and is activated following the proteolytic removal of a 14-amino-acid residue peptide from the NH_2 terminus of the protoxin. Proteolysis can result from the action of *C. perfringens* proteases, such as the λ-toxin, or the effect of trypsin in the gut. Among the symptoms provoked by ε-toxin are increased intestinal permeability, lung edema, and excess pericardial fluid. Its most striking effect is on the kidneys, which become swollen and hyperemic or, in sheep, pulpy a few hours before death. As immunity can be conferred by vaccination with a toxoid preparation (90), the gene for ε-toxin, *etx*, represented a major target for biotechnologists. This gene has recently been cloned and sequenced to produce a second-generation veterinary vaccine (207a), and preliminary mapping studies indicate a plasmid location (25b).

ι-Toxin

The ι-toxin is the last of the four major lethal toxins produced by *C. perfringens* and is normally associated with type E strains (Table 1). It provokes necrosis and, like ε-toxin, is reportedly produced from a protoxin by proteolysis (173). The toxin was purified and found to comprise two distinct polypeptide chains which acted synergistically in mouse lethality and dermonecrosis assays (203). Subsequent studies showed the smaller polypeptide to possess ADP-ribosylating activity, and actin was one of the substrates modified (190). The heavy chain did not appear to have enzyme activity, nor did it influence the activity of the smaller polypeptide, it is probably required for targeting ι-toxin to sensitive cells. Nothing is known about the genetics of ι-toxin production, and much important work remains to be done. In particular, it will be interesting to see whether,

like diphtheria toxin, both components are encoded by the same gene.

θ-Toxin

All five types of *C. perfringens* produce a lethal hemolysin, θ-toxin, which is also known as θ-hemolysin, perfringolysin O, or the thiol-activated cytolysin. The structural gene, *pfoA*, has recently been cloned, sequenced, and mapped (24, 189, 214, 215), and related genes have been detected in other clostridial species (213a). When introduced into *E. coli*, a λ phage bearing the *pfoA* gene exhibited marked instability and, although an intact coding sequence was recovered, the promoter and regulatory region were missing (214, 215). Consequently, little could be deduced about possible control mechanisms governing expression. However, recently the *pfoA* gene, and the entire region upstream of this gene, were cloned onto a low-copy-number plasmid (189). Subcloning and sequence studies showed that approximately 1.6 kb upstream of *pfoA* there was a second gene, designated *pfoR*, which acted to enhance the expression of *pfoA*. The deduced amino acid sequence of the *pfoR* gene product had many of the motifs typical of DNA-binding proteins. It appears that the PfoR protein acts as a *cis*-dominant positive activator of *pfoA* gene expression (189). To our knowledge, this is first time that researchers have characterized a gene that is directly involved in the regulation of a structural gene responsible for the production of an extracellular toxin in *C. perfringens*.

The primary sequence of θ-toxin, deduced from the *pfoA* gene sequence, revealed a 494-residue preprotein from which a 28-residue signal peptide is removed on secretion to the medium. Mature θ-toxin has a predicted molecular weight of 52,469 and, when purified from *C. perfringens* or *E. coli*, appears as a 54,000-Da species (214, 215).

θ-toxin is a member of a family of thiol-activated cytolysins produced by a diverse group of gram-positive bacteria including the *Bacillus*, *Streptococcus*, *Clostridium*, and *Listeria* genera. These cytolysins use cholesterol as a receptor and form large pores in target cell membranes. They are reversibly inactivated by oxidation. To date, the primary structures of five of these cytolysins (alveolysin, listeriolysin, streptolysin, pneumolysin, and perfringolysin) have been deduced from a cloning and sequencing approach. Despite variations in size, there are extensive regions of sequence identity, some of which correspond to shared epitopes (51). An interesting stretch of conserved sequence, an undecapeptide, ECTGLAWEWWR, encompassing the unique cysteine residue, is found near the COOH terminus. For listeriolysin, replacement of the cysteine residue by site-directed mutagenesis led to reduced activity, whereas substitution of the tryptophan residues resulted in complete inactivation and concomitant attenuation of *Listeria monocytogenes*. This study clearly demonstrated the essential role of this domain in cytolysis (128).

The θ-toxin plays a role in the tissue necrosis associated with *C. perfringens* gas gangrene and is responsible for the depletion of polymorphonuclear leukocytes in the affected zone. Low concentrations of θ-toxin cause altered polymorphonuclear leukocyte morphology, metabolism, and migration (200).

Neuraminidase

All *C. perfringens* types produce a neuraminidase, or sialidase, which cleaves acylneuraminic acid residues from glycoproteins, glycolipids, and oligosaccharides and may play a role in disease by destroying receptor molecules on cell surfaces or by disrupting connective tissue (125). In early studies, three distinct forms of the enzyme (64, 105, and 310 kDa) were found by gel filtration (170). The larger two of these enzyme forms appeared to correspond to different multimers and appeared to be associated both physically and genetically with hemagglutinins (169, 171). A gene, *nanH*, encoding neuraminidase activity has been cloned (161), but on sequencing it was shown to correspond to a previously undetected minor form (160). The *nanH* gene product is a cytoplasmic protein of 42,770 kDa which shares some sequence homology with other neuraminidases of bacterial and viral origin but differs from the major form of the enzyme with respect to its size and activity profile (160). Interestingly, when the highly homologous *nanH* gene from *Clostridium sordellii* was cloned, DNA sequence analysis showed that the enzyme was synthesized as a preprotein which is probably processed by a lipoprotein-specific signal peptidase (174). The putative processing site includes the cysteine residue which is predicted to be covalently modified by attachment of lipids during maturation. Presumably, the lipid-modified enzyme is anchored in the cytoplasmic membrane. Although the second amino acid residue of the *C. perfringens* NanH protein is a cysteine, to which the lipid should be attached, no signal peptide is predicted by the DNA sequence, suggesting that the gene may have undergone a rearrangement. It may be significant in this respect that a lysogenic bacteriophage has integrated into the chromosome near *nanH* (24, 25). Clearly, further work is required to clarify the nature and number of neuraminidases present in *C. perfringens* and to evaluate their role in pathogenesis.

μ-Toxin

The μ-toxin is a hyaluronidase which degrades hyaluronic acid, an important constituent of connective tissue, to release glucosamine (125). It is a common contaminant in commercial preparations of neuraminidase (27). Recently, the *nagH* gene was cloned and sequenced (25a) and shown to encode a β-*N*-acetylglucosaminidase which could correspond to μ-toxin. The gene is present in all *C. perfringens* isolates examined, and its product is a 97,000-Da secreted protein. Truncated derivatives of *nagH*, containing as little as 421 of the 943 codons, encode enzymatically active products; this suggests that the enzyme may consist of several domains. Although definitive identification is still required, it is conceivable that the primary role of the NagH protein/μ-toxin is in cell wall biosynthesis or autolysis and that its action as a virulence factor is secondary.

Other Toxins

The remaining toxins produced by different *C. perfringens* strains are δ-toxin, a hemolysin; κ-toxin, a collagenase; λ-toxin, a protease; ν-toxin, a nuclease; and the eta and gamma toxins, whose existence is dubious. Apart from the observation that the λ-toxin, or caseinase, gene is carried on a small plasmid in one type B strain of *C. perfringens* (14), nothing is known about their genetics. An extracellular endonuclease from one strain of *C. perfringens* has been characterized recently, but it is unclear whether this enzyme is the ν-toxin (140a). There has been little biochemical progress on the other toxins since the last comprehensive review (125).

TABLE 8. Principal features of the *C. perfringens* enterotoxin[a]

Property	Characteristics of enterotoxin
Primary gene product	320 amino acids, mol wt 35,391
Activated enterotoxin	24 amino acids removed from NH_2 terminus by trypsin (in vitro); new mol wt 31,472
pI	4.3
Stokes radius	2.6 nm
Heat stability	Unstable inactivated ≥53°C
SDS binding	Anomalous, 0.39 g/g of protein
Solubility	Low (3.94 ± 0.22 mg/ml at pH 7.0 and 25°C)
Free thiol groups	One
Domain structure binding	Two domains, N terminal (cytotoxic) and C terminal (receptor)
Biological activity	Pore formation; altered permeability, ion flux, inhibition of macromolecular synthesis; Ca^{2+}-mediated cytoskeletal disintegration; cell lysis

[a] According to Granum, McClane, and coworkers (55, 68, 76, 121–123, 126).

ENTEROTOXIN

Of the major virulence factors produced by *C. perfringens*, the enterotoxin is one of the best studied. There is an enormous literature dealing with the enterotoxin, and it is beyond the scope of this article to summarize all the available knowledge. Instead, the molecular genetic aspects will be given priority, and the reader should refer to several recent comprehensive reviews for additional details (55, 59, 121, 125).

C. perfringens is the etiological agent of two very different types of food poisoning affecting humans and is the third most important source of food-borne disease after *S. aureus* and *Salmonella* species (29, 86). Some type A strains are associated with a mild form of food poisoning, resulting from enterotoxin production, whereas type C strains are responsible for necrotic enteritis, a rare but often deadly intestinal disease provoked by production of the β-toxin.

C. perfringens food poisoning occurs following ingestion of heavily contaminated food, usually meat products containing more than 10^6 CFU/g. *C. perfringens* cells sporulate in the small intestine and concomitantly generate large quantities of enterotoxin. This can result, 12 to 24 h later, in intense nausea, diarrhea, and, more rarely, vomiting, with the occasional fatality amongst the elderly and debilitated. Infected individuals generally recover rapidly and are unaware of the origin of their malaise. In addition, it has also been suggested that *C. perfringens* enterotoxin may be involved in antibiotic-associated diarrhea (16, 94).

Biochemical Properties of Enterotoxin

The purification of large quantities of the enterotoxin (61, 126) had important consequences for the elucidation of its mode of action and functional organization, the determination of its amino acid sequence, and ultimately the cloning of the structural gene. The principal properties of the enterotoxin are summarised in Table 8. It is clear that the enterotoxin accumulates in large quantities intracellularly, where, owing to its limited solubility, it can form inclusion bodies (102). Following lysis of the bacteria in the gut, the toxin is liberated and, like the ε-toxin, processed to a more active form by trypsin (55, 157).

The current model for enterotoxin-mediated cytotoxicity involves two distinct phases which differ in their dependence on Ca^{2+} ions (121–123). In the initial, Ca^{2+}-dependent step, enterotoxin inserts into the brush border membrane of epithelial cells, where it forms a complex with a two-subunit protein receptor consisting of 50- and 70-kDa polypeptides (225). Membrane insertion is probably accompanied by a radical conformational change (58), whereas complex formation is strongly correlated with cytotoxicity (122). Intestinal cells thus affected display altered permeability and lose low-molecular-weight metabolites and ions. These early lethal events are Ca^{2+} independent and have pleiotropic inhibitory effects on the synthesis of macromolecules (76). However, the presence of Ca^{2+} provokes the second phase of enterotoxin cytotoxicity, and this involves morphologic damage and more extensive permeability alterations, resulting in the loss of larger solutes. One possible consequence of the increased influx of Ca^{2+} is cytoskeletal collapse, which will culminate in cell lysis (57, 123). *C. perfringens* enterotoxin is quite distinct from other intestinally active toxins and may be considered as the prototype for a new class of membrane-seeking cytotoxins (121).

The availability of large amounts of highly purified enterotoxin allowed the amino acid composition and sequence to be determined (156), and this not only provided important biochemical information but also facilitated the subsequent cloning of the gene. Curiously, other workers (101) reported the purification of a second "enterotoxin" from the coatless spore mutant, 8-6. As this strain is known to produce copious amounts of bona fide enterotoxin, the findings of this study should be interpreted with caution.

Molecular Genetics of Enterotoxin

Cloning of the enterotoxin structural gene. Two research groups have independently designed oligonucleotide probes, based on the protein sequence of the enterotoxin, and used them to screen gene banks (83, 218). Both groups identified clones which contained fragments of the enterotoxin gene, *cpe*, and in one case these overlapped sufficiently to permit the elucidation of the complete nucleotide sequence (218). Other workers used antibodies raised against enterotoxin to screen a *C. perfringens* expression library prepared in λgt11 and isolated a clone producing an immunologically cross-reacting hybrid protein (69). Despite being isolated by three different research groups, an intact *cpe* gene has not yet been cloned, and, as a consequence, no data are available concerning expression in any other hosts. Furthermore, nothing is known about the transcriptional control of *cpe*, and the promoter has yet to be identified. This is important as there are several reports which suggest low-level expression in the absence of sporulation (53, 60), although it is clear that the levels of enterotoxin production are increased considerably following the onset of sporulation.

Structure-function relationships. Comparison of the deduced amino acid sequence with that determined experimentally (156, 218) revealed reasonably good agreement, with

the exception of a small peptide which had proved refractory to protein sequence analysis. The native enterotoxin thus contains all 20 amino acids and comprises 320 residues to give a molecular weight of 35,391 (218) (Table 8).

The current structural model for enterotoxin suggests a two-domain organization with the N-terminal region required for cytotoxicity while the C terminus interacts with cell membrane receptors, one of which is a 50-kDa protein species (225). Results of a UV differential spectroscopy study of purified enterotoxin support this model (62), and recent independent confirmation was obtained in studies with a recombinant C-terminal fragment (121). Hanna and McClane (68) convincingly demonstrated that residues 171 to 320 of the enterotoxin were sufficient for receptor recognition and binding. Although this interaction was irreversible, it was not accompanied by membrane insertion or any of the biological activities associated with enterotoxin. This suggests that the NH_2-terminal domain is responsible for cytotoxicity. It is clear that our understanding of enterotoxin function and molecular organization will benefit from further structural studies.

Gene location and molecular epidemiology. The cloned *cpe* gene has proved to be a useful tool in molecular epidemiological studies of numerous *C. perfringens* isolates from various sources. In an initial survey in which specific oligonucleotide probes were used to screen a collection of 98 randomly chosen strains isolated from the feces of farm animals, only 6 isolates that hybridized with the probe were detected (218). This approach was later extended to include 245 strains, isolated from food and feces, associated with 186 food-poisoning outbreaks of known *C. perfringens* etiology (217). In this study 59% of the isolates were cpe^+ by hybridization, and the authors concluded that the remainder were probably part of the normal intestinal flora and had been mistakenly identified as the cause of the food poisoning. In addition, comparative studies suggested that hybridization with a *cpe* probe was a more reliable method than enzyme-linked immunosorbent assay (146) for detecting potentially enterotoxigenic *C. perfringens* strains (217). The value of this approach was also demonstrated in studies on an outbreak of porcine diarrhea due to a type A *C. perfringens* strain. In this example the use of a probe clearly ruled out enterotoxin production as the cause of the disease (216).

By using a similar approach, the relationship between enterotoxigenicity and plasmid profile was examined, and it was found that the *cpe* gene was not associated with a widely distributed series of small plasmids (149). The results suggested a chromosomal location for this gene. By means of the genome mapping strategy outlined above, as well as intragenic probes, the *cpe* gene has been located to a single locus between the *rrnF* and *malG* genes on the chromosome of the enterotoxigenic type A strain 8-6, a derivative of NCTC 8798 (175a). It has long been known that enterotoxin production is not confined to type A strains but also occurs in some type C and D isolates (55), and these combined observations suggest that the *cpe* gene may be located on a mobile genetic element. Clearly, this requires further investigation and will yield some interesting results. Finally, it is worth repeating that despite the relatively limited distribution of the *cpe* gene, *C. perfringens* is a major cause of food-borne disease (29, 86).

Enterotoxin and Sporulation: Facts and Myths

Now that it is clear that there is a single *cpe* gene, with a relatively narrow distribution, it is easier to reevaluate the relationship between enterotoxin production and sporulation and to reconcile some contradictory data. It is often mistakenly believed that enterotoxin is a component of the spore coat of *C. perfringens* (41) or even that it corresponds to excess, unincorporated spore coat material (93); these observations are the cause of much confusion. As virtually all Ent^- strains are capable of sporulation, it is evident that the enterotoxin is not an essential spore component.

In a thorough genetic study, Duncan et al. (36) isolated a group of sporulation mutants of strain NCTC 8798, which contains the cpe^+ gene, and evaluated enterotoxin production. They found that all *spo* mutants were defective in experimental enterotoxemia, whereas some of the oligosporogenous mutants, and mutants blocked in stages III, IV, and V of sporulation, produced reduced amounts of enterotoxin. This was rightly interpreted as indicating a clear-cut relationship between sporulation and enterotoxin production. However, the low levels detected in known Ent^+ strains growing in batch (60) or chemostat (53) cultures suggest that the *cpe* gene may be expressed constitutively from a weak promoter during the exponential phase of vegetative growth. Presumably the levels of enterotoxin thus produced are below the levels of detection of the techniques that were used previously (36) with the Sp^- mutants of NCTC 8798.

Following the onset of sporulation at stage II, enterotoxin synthesis is induced and, according to Granum et al. (60) precedes the appearance of heat-resistant spores by several hours. Given that mutants blocked in the later stages of sporulation (III to V) produce the toxin, it seems likely that its gene is transcribed from a developmentally regulated promoter which requires the product of an unknown (stage I or II) *spo* gene(s) for activation. Thus the *cpe* gene may play no role in sporulation per se but simply be subject to coordinate control in response to an environmental signal. Such regulation is common place in the related organism *B. subtilis*, in which, for instance, the *spo0A* gene controls competence development as well as sporulation initiation (204). The availability of the cloned gene will allow this hypothesis to be tested and should shed further light on the complement of regulatory factors available in *C. perfringens*.

Although some workers have convincingly showed the enterotoxin to be localized in the cytoplasm, by immunostaining of thin sections of sporulating cells (172, 222), others claim that it is actually a component of the spore coat (41). Solubilization and manipulation of spore proteins are notoriously difficult and, together with the tendency of the enterotoxin to aggregate, render localization studies hazardous. Recently, a Western immunoblotting analysis of spores purified from Ent^+ and Ent^- *C. perfringens* strains clearly showed that enterotoxin was not a major structural component of the spore coat but may have been trapped between the core and coat layers (175). However, a 48-kDa protein which strongly cross-reacted with antiserum raised against enterotoxin was shown to be a minor component of the spores irrespective of the enterotoxicity of the parent strains. As antibodies to this spore coat constituent also recognize enterotoxin, it would appear that the two polypeptides have common epitopes. Although the functional significance of this is obscure, it does provide a logical explanation for the results of localization studies published previously (41).

In conclusion, high levels of enterotoxin are produced in the cytoplasm after activation of the *cpe* gene by transcriptional factors which also control sporulation genes. Accu-

mulation of large quantities of enterotoxin can result in aggregation, inclusion body formation, and trapping of small amounts during spore biogenesis.

FUTURE PERSPECTIVES

The principal aim of this review was to summarize recent advances in our knowledge and understanding of the molecular genetics and pathogenicity of *C. perfringens*. It should be clear to the reader by now that the development of genetic tools and systems during the last 5 years has prepared the way for a detailed analysis of the diseases associated with this obligate anaerobe. Now that we have at our disposal the genes for several virulence factors, as well as the means of reintroducing them into *C. perfringens*, it should be relatively simple to construct a series of isogenic mutants by gene replacement. Alterations in the pathogenic spectrum could then be evaluated by using a suitable animal model.

It is conceivable that after inactivation of the genes for the established virulence determinants, additional factors which contribute to pathogenesis will be discovered. In this respect it would be wise to follow closely the developments with related pathogens such as *B. cereus*, *Streptococcus pneumoniae*, and *Listeria monocytogenes*, as these could provide fresh insight and new leads. It is interesting that in *L. monocytogenes* a phosphatidylinositol phospholipase has recently been discovered which allows the organism to escape from one cell and infect another (31c). A similar enzyme is produced by *Clostridium novyi*, and it is quite possible that some *C. perfringens* strains also possess this activity. Another probable virulence determinant which has not yet been examined is capsule production. Many *C. perfringens* strains isolated from individuals with infections are encapsulated, and this clearly enables the organism to resist or escape phagocytosis. Although this property is often lost after repeated passage in the laboratory, it should be possible, by transposon mutagenesis, to identify the genes involved in capsule biosynthesis.

One aspect which has not been treated here is the biotechnological potential of *C. perfringens* and the development of attenuated, or avirulent, mutants that could render the organism more attractive to industry. *C. perfringens* has many properties of industrial interest, among the more obvious being its short doubling time (ca. 20 min), high growth yield, potential to secrete proteins of interest, ability to grow anaerobically (thus reducing fermentation costs), and relative aerotolerance (which enables genetic manipulation to be performed without recourse to anaerobic incubation chambers). One area in which *C. perfringens* could make a valuable biotechnological contribution is as a host for high-level expression of large genes rich in dA+dT. Among the obvious candidates are other clostridial genes such as those encoding the tetanus toxin, the botulism toxins, or the *C. difficile* cytotoxins, as well as those of unrelated organisms such as *Plasmodium* spp. or trypanosomes. Another aspect which could be developed in the 1990s is the construction, by reverse genetics, of strains suitable for use as live veterinary vaccines.

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